UNIVERSIDAD D CÓRDOBA

Tesis Doctoral

Marcadores moleculares de ADN: análisis de la variabilidad, relaciones genéticas y mapeo en olivo (*Olea* europaea L.)

Trabajo realizado en el Instituto de Investigación y Formación Agraria, Pesquera y Alimentaria (IFAPA)- Centro Alameda del Obispo de Córdoba y en el Departamento de Genética de la Universidad de Córdoba para optar al grado de Doctor por la licenciada en Biología:

Mª del Carmen Domínguez García

Córdoba, julio 2012

Directores: Raúl de la Rosa Navarro
Angjelina Belaj

TITULO: *MARCADORES MOLECULARES DE ADN: ANÁLISIS DE VARIABILIDAD, RELACIONES GENÉTICAS Y MAPEO EN OLIVO (OLEA EUROPEA L.)*

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MARCADORES MOLECULARES DE ADN: ANÁLISIS DE LA VARIABILIDAD, RELACIONES GENÉTICAS Y MAPEO EN OLIVO (*Olea europaea* L.)

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INFORMAN:

Que el trabajo titulado **"Marcadores moleculares de ADN: análisis de la variabilidad, relaciones genéticas y mapeo en olivo (*Olea europaea* L.)"** ha sido realizado por **Dña. Mª del Carmen Domínguez García** bajo su dirección, se considera finalizado y reúne los requisitos necesarios para su exposición y defensa como Tesis Doctoral en el Departamento de Genética de la Universidad de Córdoba.

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UNIVERSIDAD DE CORDOBA

Doctorado

omisión

TÍTULO DE LA TESIS: Marcadores moleculares de ADN: análisis de la variabilidad, relaciones genéticas y mapeo en olivo (*Olea europaea* L.)

DOCTORANDO/A: Mª del Carmen Domínguez García

ESCRITO RAZONADO DEL TUTOR DE LA TESIS

(Ratificando el informe favorable del director. Sólo cuando el director no pertenezca al Departamento).

Dña. Adoración Cabrera Caballero, como tutora de la Tesis Doctoral de Mª del Carmen Domínguez García, ratifica el informe favorable dado por los directores de la tesis D. Raúl de la Rosa y Dña. Angjelina Belaj para su exposición y defensa.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, julio de 2012

Firma del tutor/a

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PUBLICACIONES OBTENIDAS DE ESTA TESIS DOCTORAL

Publicaciones en revistas internacionales de SCI

Dominguez-Garcia MC, de la Rosa R, Laib M, Belaj A. Characterization and identification of the main Algerian olive cultivars by molecular markers (2012), Journal of Horticultural Science and Biotechnology, 87 (2); 95-100.

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Belaj A., Dominguez-García M.C., Atienza S.G., Martin Urdiroz N., de la Rosa R., Satovic Z., Martin A., Kilian A., Trujillo I., Valpuesta V., del Rio C. Developing a core collection of olive (*Olea europaea* L.) based on molecular markers (DarTs, SSRs, SNPs) and agronomic traits (2012), Tree Genetic and Genomes 8, 365-378.

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MC Dominguez-Garcia, A Belaj, R de la Rosa, Z Satovic, A Kilian, A Martin, S Atienza, 2012. Utilidad de marcadores DArT en olivo. XIII Congreso Nacional de Ciencias Hortícolas. Acta de Horticultura, 60 pp. 125. Almería (España).

Resumen

El olivo (*Olea europaea* L.) es uno de los cultivos más antiguos de la cuenca mediterránea y cuenta con un gran patrimonio genético. Sin embargo, la tendencia actual en la mayoría de los países olivareros es hacia la utilización de pocas variedades muy populares. Por tanto la conservación de esta diversidad genética resulta de vital importancia para evitar su pérdida. Durante las últimas décadas, se han realizado importantes trabajos de prospección, recolección, caracterización y evaluación de las variedades más importantes de olivo. La conservación y el estudio de los recursos genéticos del olivo implican el establecimiento de Bancos de Germoplasma, basados en colecciones en campo, como ocurre en el resto de especies frutales. El primer aspecto a tener en cuenta en el manejo de estas colecciones es la correcta identificación del material existente, así como de las entradas que se van incorporando de manera sucesiva. En el caso del olivo, la presencia recurrente de sinonimias (la misma variedad con distintos nombres) y homonimias (distintas variedades con el mismo nombre), hace que se acentúe la necesidad de una correcta identificación varietal.

El Banco de Germoplasma Mundial de Olivo (BGMO) de Córdoba es uno de los dos bancos de referencia Internacional. Incluye más de 450 cultivares de 17 países distintos, lo que representa un porcentaje elevado de la variabilidad de la especie en la cuenca mediterránea. La correcta identificación de ésta y otras colecciones de germoplasma de olivo así como el estudio de la variabilidad genética son principalmente realizadas por marcadores moleculares. También tiene un creciente interés la utilidad de los mismos en la mejora genética. En el presente trabajo se han utilizado tres tipos de marcadores moleculares (SSRs, SNPs y DArTs) para la identificación, estudios de variabilidad genética y mapeo en germoplasma asociado al BGMO.

En un primer apartado se ha realizado la identificación de 14 nuevas variedades argelinas, recientemente introducidas en el BGMO, con marcadores moleculares (SSRs y SNPs). La comparación de los perfiles de estos marcadores con 5 variedades de Argelia, Túnez y Marruecos, previamente

incluidas en el BGMO, indicó que estas variedades difieren de ellas y del resto y su introducción enriquecerá el BGMO con material vegetal de este país. Aunque se han detectado algunos casos de homonimias en las variedades argelinas, la alta variabilidad genética observada es un indicio de la riqueza del su germoplasma.

Posteriormente se ha descrito la puesta a punto de los primeros marcadores moleculares de alto rendimiento para el olivo, los denominados DArTs. Estos marcadores no necesitan un conocimiento previo de la secuencia de ADN. Aunque son dominantes, tienen una alta reproductividad (99,8%) y un bajo coste. Se han utilizado para identificar de manera inequívoca una muestra de 62 variedades pertenecientes al BGMO. El dendograma construido en base a ello ha confirmado su agrupación por origen geográfico, previamente observada con otros marcadores moleculares. También se ha demostrado la utilidad de estos marcadores de alto rendimiento en la construcción de un mapa de ligamiento en una progenie de las variedades ‘Picual’ y ‘Arbequina’. Se han obtenido un total de 23 grupos de ligamiento para cada parental. Además algunos microsatélites, también incluidos en dicho mapa, han permitido establecer relaciones entre grupos de ligamiento de ambos parentales.

Por último, la variabilidad existente en el BGMO se ha evaluado con marcadores DArT, microsatélites, SNPs y características agronómicas, para construir la primera colección nuclear de dicho Banco. Se han obtenido cinco colecciones nucleares, que contenían desde 18 hasta 68 variedades. De todas ellas, la colección nuclear de 68 variedades parece la más indicada para estudios de conservación, dado que retenía todos los alelos y caracteres analizados. Por otro lado, la colección nuclear de 36 variedades se reveló como la más adecuada para estudios de mejora, dada la elevada distancia genética media y la buena representación de las distintas regiones del Mediterráneo en un número relativamente pequeño de variedades.

Summary

Olive (*Olea europaea* L.) is one of the oldest trees cultivated in the Mediterranean Basin and it includes a high genetic patrimony. However, in most olive growing countries, olive orchards are composed by a very reduced number of well known cultivars. In this sense, the preservation of olive genetic patrimony is of vital importance for avoiding the possible erosion. During the last decades, considerable prospections, recollections, characterization and evaluation surveys, have been performed on the main olive cultivars.

Similarly to other fruir species, the conservation and the study of olive genetic resources implies, the establishment of 'ex situ' collections (Germplasm Banks). The identification of existing plant material as well as of new accessions continously introduced in the collections is a priority task for their correct management. In olive, the presence of many synonyms (the same variety with different names) and homonyms (different varieties with the same name) makes the identification highly needed.

The World Olive Germplasm Bank (WOGB) in Cordoba, is one of the two international collections of reference. It includes more than 450 cultivars from 17 different countries thus representing a high percentage of the variability of species in the Mediterranean Basin.

Molecular markers have mostly been used in olive for correct identification of germplasm collections as well as for genetic diversity studies. Their utility for olive breeding has being of great interest. In the present work, three types of molecular markers (SSRs, SNPs and DarTs) have been used for identification, mapping studies and genetic variability of plant material related to the WOGB.

The first part of this work deals with the identification of 14 new Algerian cultivars, recently introduced into the WOGB, by means of SSR and SNP markers. The comparison of their molecular profiles with 5 existing cultivars of Algerian, Tunisian and Marocco origin showed that they were different, enriching so the WOGB with plant material from this country. Our study showed the great variability of Algerian germplasm as well as some cases of homonyms.

The setting up of the first high throughput marker for olive (DArTs) has been further described. These markers do not require prior sequence information. They are dominant makers, with a high reproducibility (99,8%) and lower cost compared to other markers. The use of these markers for the correct identification of 62 cultivars belonging to WOGB has been evaluated. In addition these markers have been differentiated 62 cultivars according to their geographic origin, separating Eastern and Western varieties from the Mediterranean ones.

The utility of DArT markers for the development of a linkage map in one progeny of Picual' x 'Arbequina'cultivars has been confirmed. A total of 23 linkage groups have been obtained per each parent. Besides, some of the SSR used allowed the stablishment of some connections with several linkage groups of both parents.

Finally, the existing genetic variability in the WOGB has been evaluated by means of DarTs, SSRs, SNPs and agronomical traits in order to develop the first core collection. Five core collections including from 18 to 68 cultivars were obtained. From these core sets, the one with 68 cultivars seems the most appropriate for conservation studies as it retained all the alleles and traits under study. On the other hand, the core 36 was found to be the most indicated one for breeding given the high genetic distance and the good representativeness of the Mediterranean regions in a realitvely small number of cultivars.

CAPÍTULO I

Introducción general

I.1. El cultivo del olivo

I.1.1. Clasificación botánica y características del olivo

El olivo pertenece a la familia *Oleaceae* compuesta por unas 600 especies y 24 géneros de árboles y arbustos (Besnard et al, 2009). Algunos de estos géneros, que constituyen esta familia, tienen interés económico u hortícola, estos son el fresno, el jazmín, el aligustre, el agracejo y la lila (Heywood, 1978).

Dentro de esta familia, el olivo se integra en el genero *Olea,* teniendo éste 33 especies (Besnard et al, 2009). La especie *Olea europaea* L. es la única que posee frutos comestibles (Rapoport, 2008). Esta especie la componen seis subespecies: *Olea europaea* subsp. *europaea* (Cuenca del Mediterráneo); *O. e. laperrinei* (distribuidos en los macizos de Hoggar en Argelia); *O. e. cuspidata* (África de Sur, Egipto, Australia, Hawai, Arabia, India y China); *O. e. guanchica* (Islas Canarias); *O. e. maroccana* (Marruecos) y *O. e. cerasiformis* (Madeira). Dentro de la subespecie *Olea europaea* subsp. *europaea*, tenemos dos variedades *O.e.* subsp. *europaea* var. *europaea,* donde se engloban todos los olivos cultivados y *O.e.* subsp. *europaea* var. *sylvestris,* a la que pertenecen los olivos silvestres o acebuches (Besnard et al, 2009). Todas estas subespecies poseen 23 pares de cromosomas, un número elevado que apunta a un posible origen alopoliploide (Bartolini et al., 2002). Por tanto, cabe esperar que pueden haberse producido cruces naturales y reorganización cromosómica entre especies afines de *Olea*. La falta de homología entre los 23 pares de cromosomas que forman su cariotipo indica su naturaleza híbrida (Falistoco y Tosti, 1996).

El hábitat del olivo se concentra entre las latitudes 30º y 45º, tanto en el hemisferio norte como en el hemisferio sur y en regiones climáticas del tipo mediterráneo, caracterizadas por un verano seco y caluroso (Barranco et al, 2008). Es una especie muy longeva, pudiendo perdurar varios cientos de años (Muñoz-Diéz, 2008; Diéz et al., 2011). Es un árbol de tamaño medio, de 4 a 8 metros de altura dependiendo de la variedad de que se trate. Su tronco es grueso, con una corteza de color grisáceo; la copa es redondeada y la

ramificación natural tiende a producir una copa bastante densa (Rapoport, 2008). Posee hojas verdes por el haz y blanquecinas por el envés. La flor es menuda y el fruto es una drupa, constituido por el endocarpo que es el hueso, el mesocarpo o pulpa, que en este caso es oleosa, y el exocarpo que es la piel.

Los principales usos del olivo son: (1) Alimentario: tanto por el aceite de oliva como por las aceitunas de mesa; (2) Madera: aprovechada en muchos países para la artesanía y utensilios de cocina; (3) Cosmético: su aceite es utilizado por sus propiedades hidratante, protectora y antienvejecimiento y (4) Ornamental: en los últimos años está siendo muy utilizado en parques y jardines tanto públicos como privados.

I.1.2. Origen e historia del cultivo del olivo

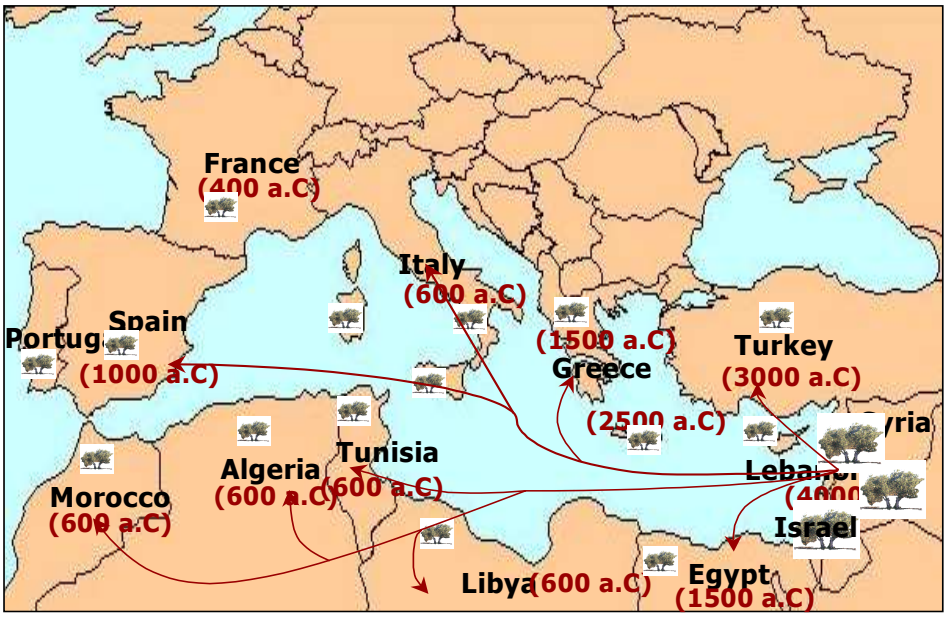
Loukas y Krimbas (1983) sitúan el momento en el que se origino el cultivo del olivo en la edad de Cobre, es decir unos cuatro mil o tres mil años antes de Cristo. Este periodo fue acortado por Zohary y Hopf (1994) que sugieren que su domesticación se produjo hace entre 5500 y 5700 años. De cualquier modo, su origen es muy antiguo, remontándose su cultivo a la Prehistoria. Se desconoce cuando comenzó su cultivo para la obtención de aceite de oliva, pero se conservan molinos primitivos de los años 4000-3500 a. C., al sur de Jordania (Ben-Thot, 1992). Se cree que los restos de endocarpo encontrados junto con los granos de cereales, dátiles y leguminosas, en las excavaciones de estos primitivos molinos, corresponden a olivos cultivados ya que evidencian la presencia de un fruto mayor que las aceitunas silvestres (Zohary y Sepiegel-Roy, 1975). Aunque no es hasta el año 2500 a. C. donde encontramos escritos sobre el aceite de oliva en Creta, estos documentos son tablas minoicas, que muestran la importancia del aceite de oliva para la corte del rey Minos (www.oleohispana.com, 2009).

El cultivo del olivo no surgió hasta el aprendizaje de la clonación, es decir, de la multiplicación asexual o vegetativa, formado este parte de una primera oleada de domesticación de árboles frutales (Zohary y Sepiegel-Roy, 1975), junto con la vid, la palmera datilera y el grano. Actualmente, en el olivo se conservan

muchos de estos métodos de propagación como son la estaca, el plantón, el asilamiento de zuecas, etc. (Caballero y del Río, 1997).

En cuanto al lugar donde se comenzó a cultivar, existen varias teorías, pero una de las más aceptadas es la de Vavilov (1949-1950), quien estableció el centro de origen del olivo cultivado en Siria e Irán. De ahí se extendió por toda la Cuenca del mediterráneo, de oriente a occidente, estando presente en todos los países e islas bañados por este mar (Figura I.1). La dirección de difusión del olivo fue paralela a las vías comerciales establecidas primero por los fenicios y los griegos, y posteriormente por los romanos (Bartolini et al., 2002). Éstos últimos jugaron un papel fundamental en el desarrollo del cultivo en la cuenca mediterránea, ya que practicaban una propagación masiva cada vez que ampliaban su imperio. A partir del siglo XV, y con el descubrimiento del nuevo mundo, se amplió el ámbito de cultivo del olivo hacia el continente americano. Más recientemente, se ha introducido el olivo en nuevas zonas como Australia (Barranco et al., 2008).

Fig I.1. Extensión del cultivo del olivo por la cuenca mediterránea

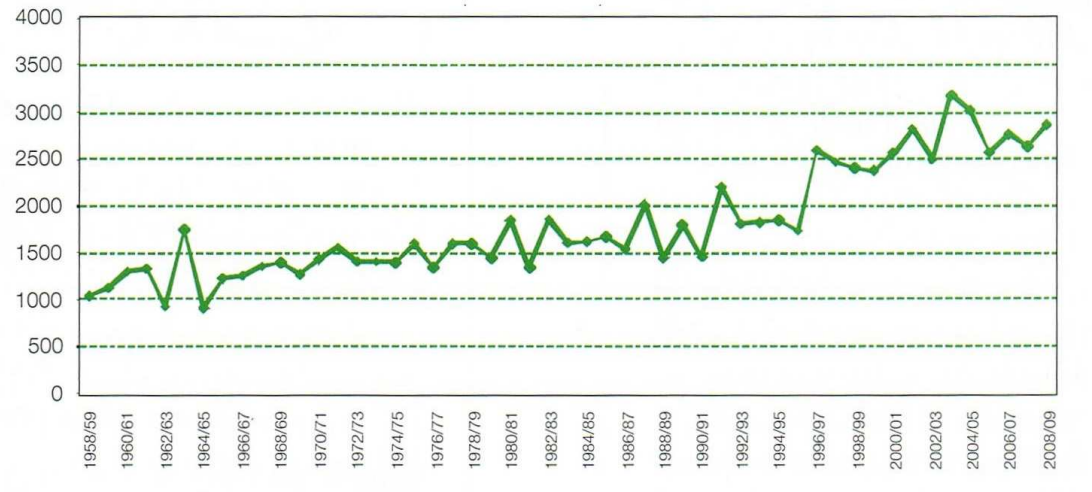


I.1.3. El olivo en el mundo actual

El olivo es la fuente principal de aceite vegetal para las culturas milenarias asentadas en la Cuenca del Mediterráneo, sin embargo es un cultivo secundario en el mundo. Se estima que existen 960 millones de olivos cultivados a lo largo del mundo, de los cuales el 98% se sitúa en los países de la cuenca mediterránea, ocupando una superficie de 10 millones de hectáreas (Civantos, 2001).

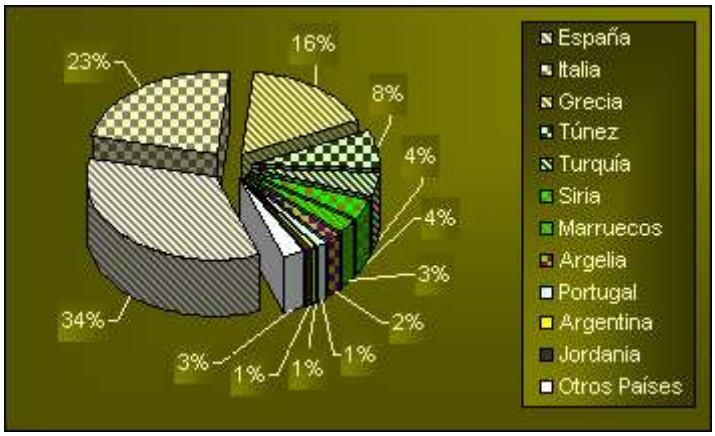
En los últimos 50 años casi se ha triplicado la producción mundial de aceite de oliva, pasando de 1032 millones de toneladas en 1958-1959 a 2866 millones de toneladas en 2008-2009 (Figura I.2). (C.O.I., 2009)

Fig I.2. Evolución de la producción mundial de aceite



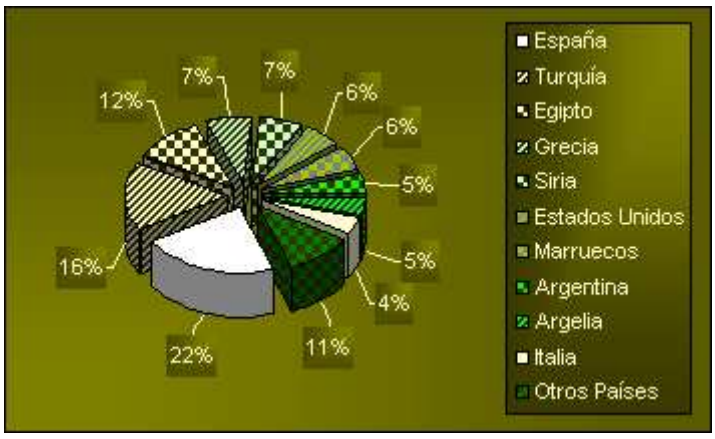
España es el principal productor de aceite de oliva, seguido de Italia, Grecia, Túnez, Turquía, Siria, Marruecos, Argelia, Portugal, Argentina y Jordania (Figura I.3). En Andalucía se concentra el 62% de la superficie de olivar de España (Barranco et al, 2008), por lo que la producción de España está muy condicionada por la aportación de esta Comunidad Autónoma.

Fig I.3. Países productores de aceite de oliva



El 10% de la producción de aceituna se consume de forma directa, es decir, preparada para su uso como aceituna de mesa. Las aceitunas que se destinan a mesa superan un millón de toneladas destacando, como es el caso del aceite de oliva, España con el 22% de la producción mundial (Figura I.4), seguida de Turquía, Egipto, y Argentina (Barranco et al, 2008).

Fig I.4. Países productores de aceituna de mesa



I.2. Bancos de germoplama

Aproximadamente, 1200 variedades han sido descritas en los principales países productores de aceite de oliva (Bartolini et al., 2005). Para la conservación y utilización de estos recursos, se procedió a la creación de

Bancos de Germoplasma en distintos países del mundo. Estos bancos son un instrumento básico para la mejora de cualquier especie.

Los bancos de germoplasma tienen dos objetivos principales. El primero de ellos es salvaguardar el patrimonio genético acumulado durante siglos de cultivo. Este objetivo es muy importante en el olivo, ya que es frecuente en los países olivareros el cultivo de muy pocas variedades principales en detrimento de las locales (Barranco y Rallo, 1983). Esto puede provocar una erosión genética que indica la necesidad de conservar el germoplasma en colecciones.

El segundo objetivo de los bancos de germoplama consiste en el estudio y evaluación de las variedades en las mismas condiciones de cultivo, de esta forma se puede conocer y explotar la variabilidad genética de la especie. El conocimiento del potencial real de cada variedad es imprescindible para conseguir una olivicultura competitiva basada en material vegetal seleccionado mediante experimentación o tras un programa de mejora genética dirigida (Caballero y Del Río, 1999).

Además de estos dos objetivos, los bancos de germoplasma tienen que suministrar material identificado a otras colecciones y/o instituciones de investigación. Para que puedan llevarse a cabo nuevos estudios.

Debido a la cantidad de material disponible y la necesidad de conservación de este, en la actualidad existen un total de 94 bancos de germoplasma distribuidos a lo largo del mundo (Tabla I.1). Estos están registrados en la base de datos mundial de la FAO, accesible a través del sitio web http://apps3.fao.org/wiews/olive/oliv.jsp.

Tab I.1. Origen y bancos de germoplasma en el mundo.

País	Número de Bancos
Albania	1
Argelia	2
Argentina	6
Australia	4
Azerbaijan	1
Brasil	1
China	2
Chipre	1
Egipto	1
España	5
Estados Unidos	2
Francia	3
Grecia	2
India	6
Irán	4
Israel	1
Italia	21
Japón	2
Jordania	1
Marruecos	6
Montenegro	1
Nepal	5
Portugal	3
Sudáfrica	1
Túnez	11
Turquía	1

I.2.1. Banco de germoplasma mundial de olivo de Córdoba

El Banco Germoplama Mundial de Olivo de Córdoba (BGMO) representa la colección más completa del mundo, incluye mas de 400 cultivares de 20 países diferentes (Caballero et al., 2005). Ello representa un porcentaje elevado de la variabilidad de la especie en la cuenca mediterránea.

La historia de este amplio reservorio genético se remonta a los años 70, cuando se inició un proyecto con un centenar de variedades de España, Italia, Turquía, Túnez y Portugal. Su desarrollo se encargó a investigadores de la FAO y el INIA, con el apoyo del COI (Rallo et al., 2005), en base a una

prospección realizada en todas las zonas olivareras de España (Barranco y Rallo, 1983) y a prospecciones e intercambios de material con la mayoría de los países olivareros.

En la primera colección, plantada a 7x7 m, llegó a contener 273 variedades, aunque tras años de trabajo de identificación, se vieron que solo se incluían 170 variedades distintas. Más tarde, en 1982, la obligación de salvaguardar nuevo material introducido y ampliar la colección llevo a plantar una nueva parcela. En 1987 se comenzó a plantar una tercera parcela, con el fin de conocer todo el potencial agronómico de los genotipos conservados, sin limitaciones de agua. El banco mantiene una cuarta parcela auxiliar para la recepción y multiplicación del material y recientemente se ha introducido la quinta parcela para hacer un reservorio de aislamiento del material del banco.

Desde la creación del banco de germoplasma, se han llevado a cabo trabajos de caracterización de las variedades que lo componen. En un principio se comenzó con la caracterización por una serie de criterios agronómicos básicos: el vigor, la producción, las características de la aceituna y la fenología (Caballero y Eguren, 1986; Caballero et al, 1990; Del Río y Caballero, 1994; Caballero y del Río, 1998; Del Río y García-Fernández, 2001; Del Río et al, 2002). Además también se ha llevado a cabo la caracterización del banco por la composición acídica. (Uceda et al, 1999; Beltrán, 2000; Uceda y Hermoso, 2001).

Desde el año 96 y gracias a un proyecto de la Unión Europea (“Conservación, caracterización, recolección y utilización de recursos genéticos del olivo”), se han llevado a cabo estudios genéticos mediante marcadores moleculares. Con el fin de conocer el patrimonio genético existente, para poder conservarlo (Belaj et al., 2001; 2003; 2004b; Sanz-Cortés et al., 2001, Martín y Rallo, 2005).

I.3. Principales tipos de marcadores y su utilización en la identificación y mejora del olivo

Como ya se ha mencionado anteriormente, existe una gran diversidad de cultivares y esto hace necesaria la conservación y el estudio de estos, por eso se crearon los bancos de germoplasma. El principal problema de las colecciones es la correcta identificación del material existente, así como de nuevas entradas. La presencia de muchas sinonimias (la misma variedad con distintos nombres) y homonimias (distintas variedades con el mismo nombre) acentúan la necesidad de la identificación varietal. Los marcadores moleculares han prestado un gran apoyo en la identificación y evaluación de la variabilidad de los cultivares de olivo existentes (Belaj et al., 2001; 2004b).

Además, el cultivo del olivo tiene una gran importancia económica y social en el área mediterránea, pero a pesar de esto, hasta hace pocas décadas, son pocos los avances que se han realizado en su mejora para la obtención de nuevas variedades (Rallo et al., 2008). La mayoría de las variedades de olivo actualmente cultivadas proceden de la selección empírica realizada por el agricultor a través de los siglos y están, en su mayoría, confinadas a su área de origen (Rallo, 2005; Barranco, 2008). En este sentido también los marcadores moleculares han sido de gran ayuda en los programas de mejora genética (De la Rosa et al., 2004).

I.3.1. Marcadores morfológicos

En un principio, antes de la aparición y utilización rutinaria de los marcadores moleculares para la descripción e identificación de variedades de olivo, se han venido utilizando un gran número de caracteres morfológicos. Pitton de Tournefort hizo la primera clasificación del olivo en 1719. Posteriormente, se han propuesto nuevos sistemas de identificación y clasificación del olivo, en concreto Ganino et al (2006), hace referencia a que ha habido 18 sistemas diferentes desde Pitton de Tournefort (1719), usándose éstos a lo largo de las décadas para la identificación de los olivares (Barranco y Rallo, 1984; Prevost et al., 1993; Tous y Romero, 1993; Cantini et al., 1999; Barranco et al., 2000;

Leva y Petruccelli, 2011; Trentacoste y Puertas, 2011). Esta evaluación permite una caracterización rápida, económica y eficaz de los genotipos, aunque es un método que esta sujeto a variables ambientales (Barranco et al., 2005).

En el caso del olivo, los órganos más frecuentes utilizados para la evaluación son las hojas, las inflorescencias, los frutos completos, los huesos y las semillas (Barranco y Rallo, 1984; Barranco et al., 2000). Actualmente, el Consejo Oleícola Internacional (C.O.I.) utiliza el sistema de clasificación de variedades descrito por Barranco et al. (2005). Además siguen siendo los únicos marcadores aceptados por la IPGRI (www.ipgri.cgiar.org/index.htm), aunque su utilidad sea frecuentemente reforzada por la utilización de marcadores moleculares (Fendri et al., 2010; Sesli et al., 2010; Sheidai et al., 2010; Belaj et al., 2011; Strikic et al., 2011).

En el BGMO han sido numerosos los trabajos que se han realizado con estos marcadores desde su creación (Caballero y Eguren, 1986; Cabellero et al, 1990; Del Río y Caballero, 1994; Caballero y del Río, 1998; Del Río y García-Fernández, 2001; Del Río et al, 2002). Siendo en la actualidad unos de los marcadores utilizados para la identificación (Del Río, comunicación personal).

I.3.2. Marcadores bioquímicos: isoenzimas

El polimorfismo de péptidos (proteínas/enzimas) se conoce a partir de 1954 (Allison, 1954), cuando se mostraron las primeras pruebas de un polimorfismo en poblaciones humanas. Posteriormente, las isoenzimas se han utilizado en plantas, siendo éstos los marcadores bioquímicos más ampliamente utilizados en mejora vegetal (Bednorz et al., 2004; Voylokov y Priyatki, 2004) La metodología necesaria para el desarrollo y utilización de este tipo de marcadores es relativamente sencilla, rápida y económica (Trujillo et al., 1995).

Las isoenzimas han sido muy utilizadas en olivo para la identificación varietal debido al alto grado de polimorfismo que exhiben. En el BGMO también han sido muy utilizadas, utilizándose tanto el polen (Pontikis et al, 1980; Rovira y Tous, 1995, Trujillo et al, 1995; Martín y Rallo, 2005) como las hojas (Ouazzani

et al., 1995; Seker et al., 2008a, b) para la extracción de las isoenzimas. Este tipo de marcadores son productos genéticos, por lo que difieren fenotípicamente, es decir, pueden tener una expresión diferencial dependiendo de factores ambientales, tipo de tejido, etc. Además se ha podido comprobar que muchas especies tienen sistemas isoenzimáticos monomórficos, lo que hace imposible su aplicación para programas de mejora genética. Por todo lo mencionado anteriormente, los mejoradores vienen haciendo uso de marcadores basados en el ADN.

I.3.3. Marcadores de ADN

Los marcadores de ADN proporcionan al mejorador unas herramientas valiosas, que tienen su origen en variaciones individuales en la secuencia común del ADN. Estos marcadores cubren gran parte del genoma, posibilitando su evaluación en fases muy tempranas y a partir de muestras mínimas, no son influenciables por el ambiente y no presentan interacciones intergénicas. A continuación se citan los marcadores más relevantes.

I.3.3.1. RFLPs ("Restriction Fragment Length Polymorphism")

El polimorfismo en la longitud de los fragmentos de restricción (RFLPs) expresa las diferencias entre individuos en sitios concretos de la secuencia de ADN que reconocen diferentes enzimas de restricción (restrictasas). La alteración del sitio diana de la enzima, puede deberse a una mutación puntual o a alteraciones de mayor envergadura, tales como delecciones o inserciones (Botstein et al., 1980). De modo que las bandas obtenidas tras la digestión con la enzima de restricción serán distintas dependiendo de si sus sitios de restricción se han mantenido intactos o no.

Este tipo de marcadores ha sido muy utilizado en orgánulos citoplasmáticos del olivo, en concreto en la mitocondria (Besnard et al., 2001a; Khadari et al., 2001; Besnard y Bervillé, 2002; Besnard et al., 2002; Bronzini de Caraffa et al., 2002) y el cloroplasto (Amane et al., 1999; Lumaret et al., 2000; Besnard et al.,

2001a; Besnard y Bervillé, 2002; Bernard et al., 2002). Además, este tipo de marcador se ha empleado en el estudio del polimorfismo del ADN ribosomal (Besnard et al., 2001b, 2007).

I.3.3.2. RAPDs ("Random Amplified Polymorphic DNA")

Los RAPDs (polimorfismo de productos amplificados al azar), fueron los primeros marcadores de ADN basados en la PCR que surgieron, son marcadores muy sencillos de analizar y por eso han sido muy utilizados en olivo. Fueron desarrollados simultáneamente en dos laboratorios distintos (Welsh y McClelland, 1990; Williamas et al., 1990). Se generan por la amplificación del ADN con un cebador de secuencia corta (8-10 bases) y aleatoria (Welsh et al., 1991), no es necesario conocimiento previo de secuenciación y utilizan como sustrato poco ADN (nanogramos). Su sencillez y bajo coste está contrapesada por sus limitaciones: herencia dominante y baja reproductividad entre laboratorios (Agarwal et al, 2008).

En olivo los RAPDs fueron los primeros marcadores de ADN utilizados para la identificación varietal (Bogani et al., 1994), empleándose posteriormente para dicho fin en colecciones de varios países, tales como Italia (Fabbri et al., 1995; Cresti et al., 1997), España (Belaj et al., 2001; 2004b; Sanz-Cortes et al., 2001, Martín y Rallo, 2005), Israel (Wiesman et al., 1998) y Australia (Mekuria et al., 1999; Guerin et al., 2002). También han sido utilizados para identificar material vegetal proveniente de viveros (Rubio y Arus, 1997; Belaj et al., 1999). Incluso se ha podido detectar la existencia de polimorfismo intra-cultivar en tres variedades portuguesas (Gemas et al., 2000) y en dos españolas (Belaj et al., 2004a). Además estos marcadores se han utilizado para estudios de filogenia (Hess et al., 2000; Bronzini de Caraffa et al., 2002) y para los primeros mapas de ligamiento (De la Rosa et al., 2003; Wu et al., 2004).

I.3.3.3. AFLPs ("Amplified Fragment Length Polymorphism")

El polimorfismo en la longitud de fragmentos amplificados aleatoriamente (AFLPs), se fundamenta en la amplificación selectiva de los fragmentos obtenidos en la digestión del ADN genómico con enzimas de restricción (Zabeaeu y Vos, 1993). Es una técnica más complicada que la de los RAPDs, ya que es necesario llevar a cabo la digestión del ADN genómico total, mediante dos enzimas de restricción. De esta forma se generan múltiples bandas que corresponden a fragmentos de distinto origen en el genoma, determinados por las enzimas de restricción empleadas, y que se pueden separar mediante geles de acrilamida en condiciones desnaturalizantes o bien mediante electroforesis capilar en un secuenciador automático. Para la generación de estos marcadores no es necesaria información previa, son generalmente dominantes y no son transferibles, como en el caso de los RAPDs, pero permiten la generación de un gran número de marcadores con pocas reacciones (Agarwal et al, 2008).

En el BGMO, este tipo de marcador se ha utilizado para estudiar la variación intra-cultivar de dos variedades españolas 'Arbequina' y 'Manzanilla de Sevilla' (Belaj et al., 2004a). Además Sanz-Cortés et al. (2003), empleó estos marcadores para estudiar la variación inter- e intracultivar presente en 38 individuos pertenecientes a 10 variedades de olivo españolas. Adicionalmente se han utilizado para la identificación de 47 variedades españolas (Martín y Rallo, 2005). También se han usado, empleando otro material distinto al del BGMO, para investigar las relaciones genéticas entre el olivo cultivado y el silvestre (Angiolillo et al., 1999; Baldoni et al., 2000). Los AFLPs se han usado en estudios de filogenia (Baldoni et al., 2006; Rubio de Casas et al., 2006). De la Rosa et al (2003) utilizó estos marcadores en el primer mapa de ligamiento del olivo y posteriormente, Khadari et al. (2010) y El Aabidine et al. (2010) también los utilizaron para nuevos mapas.

I.3.3.4. SCARs ("Sequence Characterised Amplified Region")

Esta técnica fue introducida por Paran y Michelmore (1993) y se basa en la amplificación de una secuencia conocida utilizando cebadores largos (20 bases), éstos pueden proceder de un fragmento obtenido por RAPDs o AFLPs que han sido clonados y secuenciados previamente y del que se van a diseñar cebadores altamente específicos. Estos marcadores han sido ampliamente utilizados en plantas de cultivo para la selección asistida por marcadores (MAS) (Zhang y Stommel, 2001).

En olivo, los marcadores SCAR han sido utilizado para la identificación de cultivares (Busconi et al., 2006) y para la trazabilidad del aceite de oliva (De la Torre et al., 2004; Pafundo et al., 2007). Además se han encontrado asociaciones entre estos marcadores y características del fruto (Mekuria et al., 2002) y asociaciones con la resistencia a determinados hongos patógenos (Hernández et al., 2001), lo que sugiere la aplicabilidad de estos a programas de mejora asistidos por marcadores.

I.3.3.5. SSRs ("Simple Sequence Repeats")

Los microsatélites son unos marcadores genéticos que se basan en la amplificación con cebadores largos de una zona previamente secuenciada. Consisten en motivos de ADN cortos, de entre una y seis pares de bases (Hamada et al., 1982) repetidos en tándem, que pueden dar lugar a polimorfismo entre individuos al cambiar el número de repeticiones y generar así fragmentos amplificados de diferente longitud. El desarrollo de estos marcadores moleculares requiere conocimiento previo de la secuencia de ADN sobre todo de la región que flanquea al microsatélite. Sin embargo, estos marcadores son muy usados en estudios genéticos del olivo porque tienen un alto polimorfismo y una alta reproductividad. Muchos autores han desarrollado microsatélites en olivo (Sefc et al., 2000; Rallo et al., 2000; Carriero et al 2002; Cipriani et al., 2002; De la Rosa el al., 2002; Díaz et al 2006; Sabino Gil et al., 2006).

En olivo estos marcadores han tenido usos diferentes, tales como discriminación de cultivares (Sarri el al., 2006; Fendri et al., 2010), estudio de relaciones genéticas entre el olivo cultivado y el silvestre (Belaj et al., 2007, 2010), construcción de mapas de ligamiento (De la Rosa et al., 2003; Wu et al., 2004; El Aabadine et al., 2010; Khadari et al.,2010), estudios de paternidad (De la Rosa et al., 2004; Mookerjee et al., 2005; Díaz et al., 2006, 2007a, b) y identificación de la composición de aceite de las variedades (Alba et al., 2009; Ayed et al., 2009).

Este tipo de marcador ha sido muy utilizado en el BGMO, tanto para identificación (Belaj et al., 2004b, Martín y Rallo, 2005) como para comprobar la capacidad discriminante de los SSR con otros marcadores (Belaj et al, 2003).

I.3.3.6. ISSRs ("Inter Simple Sequences Repeats")

Los ISSRs son fragmentos de ADN que varían entre 100-3000 pares de bases diseñados a partir de microsatélites, donde se obtienen simultáneamente entre 10 y 60 fragmentos que se separan en un gel mediante electroforesis. Esta técnica fue descrita por Zietkiewicz et al. en 1994.

Los ISSRs se han aplicado al análisis filogenéticos del complejo *Olea europaea* y en la identificación de cultivares. En estudios sobre la estructura de este complejo en las Islas Macaronesias, se han visto, con los ISSRs y con RAPDs, que las poblaciones de Madeira y Canarias no forman un grupo monofilético, sino que hay dos eventos diferentes de dispersión de *Olea* en cada una de las islas (Hess et al. 2000). En otro estudio llevado a cabo con los marcadores ISSRs, Vargas y Kadereit (2001) confirmaron el estado salvaje de algunas poblaciones de olivos de la Península Ibérica. Además, estos marcadores se han usado para distinguir 10 variedades italianas, extrayendo el ADN del fruto (Pasqualone et al., 2001) y para estudios de variabilidad intra-cultivar de 201 accesiones de 11 cultivares portugueses (Gemas et al., 2004).

I.3.3.7. SNPs ("Single Nucleotide Polymorphism")

El SNP es un polimorfismo originado por variaciones de un solo nucleótido en la secuencia de ADN. Dichas variaciones pueden consistir en inserciones o delecciones que afectan a un único par de bases (comúnmente conocida como indels), también pueden darse sustituciones de un nucleótido por otro distinto. Los SNPs se han revelado como la mayor fuente de polimorfismo en el genoma humano (Kwok et al, 1996; Kruglyak, 1997; Collins et al., 1998), siguiendo esta misma tendencia en todos los organismos vivos (Ganal et al., 2009). Debido a su abundancia a lo largo del genoma, junto con la necesidad de desarrollo de marcadores de alto rendimiento, podría ser uno de los sistemas de identificación usados en el futuro.

El desarrollo de estos marcadores requiere un gran conocimiento de la secuencia del genoma, por este motivo, en el olivo hasta hace poco no se han podido identificar. En el proyecto OLEAGEN, donde se enmarca esta tesis, se han generado gran cantidad de éstos, pero todavía se está desarrollando una plataforma para poder ser utilizados. Reale et al. (2006), fue el primero en desarrollar SNPs en olivo, en concreto fueron 8, que luego se testaron en un conjunto de variedades para llevar a cabo su identificación, dentro de estas variedades algunas pertenecían al BGMO. Posteriormente, se han realizado más estudios con nuevos SNPs y con los publicados por Reale et al. (2006) (Muleo et al., 2009; Santos Macedo et al., 2009; Hakim et al., 2010)

I.3.3.8. DArTs ("Diversity Array Technology")

DArTs es un método para el análisis del polimorfismo del ADN. Fue desarrollado por primera vez en arroz (Jaccoud et al., 2001), aunque ha sido aplicado a más de 60 especies (www.diversityarrays.com). Este tipo de tecnología, para el análisis genético, ofrece un coste bajo, un alto rendimiento y un sistema robusto. Estos marcadores no tienen la necesidad de tener conocimiento previo de la secuencia y por lo tanto, ofrecen una alternativa interesante para el olivo. De hecho esta tecnología ha sido aplicada con éxito

en diferentes especies, con poca información genética como la avena (Tinker et al., 2009), la banana (Risterucci et al., 2009), el centeno (Bolibok-Bragoszewska et al., 2009), la soja (Mace et al., 2008) o incluso en especies silvestres, tales como Hordeum chilense (Rodrigues-Suarez et al., 2012). En las especies leñosas, la tecnología DArT solo se ha utilizado en la banana (Amorim et al., 2009; Hippolyte et al., 2010; Risterucci et al., 2009), la yuca (Xia et al., 2005; Hurtado et al., 2008), el eucalipto (Sansaloni et al., 2010) y la manzana (Schouten et al., 2011) para estudios de variabilidad genética y mapeo. Por todo esto, las aplicaciones potenciales de los marcadores DArT incluyen: caracterización de germoplasma, mapeo genético, detección de genes, mejora asistida por marcadores y búsqueda de cambios de metilación en el genoma.

I.4. Bibliografía

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CAPÍTULO II

HIPÓTESIS Y OBJETIVOS

II.1. Hipótesis

El olivo es una especie, ampliamente cultivada en el sur de Europa y en particular en la cuenca del Mediterráneo (Civantos, 2001).

Desde hace cientos de años, el cultivo del olivo ha sido de gran importancia, desde el punto de vista económico, social y cultural en España, siendo el primer productor de aceite de oliva en el mundo, concentrándose este cultivo principalmente en Andalucía, donde en algunas provincias de esta comunidad es la principal o la única actividad económica relevante (Barranco et al., 2008).

A pesar de todo lo mencionado anteriormente, el conocimiento genético que se tiene del olivo es muy limitado, pudiendo deberse a su elevado número de cromosomas (2n=46).

En una industria en plena expansión, como es el caso de la olivarera, resulta esencial la caracterización inequívoca de las variedades de olivo y la conservación de estas variedades en bancos de germoplasma. Este punto cobra especial importancia en el olivo, ya que la diversidad genética de esta especie es enorme, habiéndose descrito unos 1200 cultivares distintos (Bartolini et al., 2005), aunque se sospecha que la identificación llevada a cabo podría ser errónea. Además, la gran mayoría de ellos proviene de la selección empírica realizada por el agricultor a través de los siglos (Rallo, 1994).

Por todo esto, se hace evidente, la necesidad de generar nuevas herramientas moleculares en olivo, que junto a las ya existentes, permitan una correcta caracterización de la variabilidad de la especie y que facilite su conservación en bancos de germoplasma.

Además de todo lo anterior, los últimos años se han planteado diversas hipótesis sobre el origen del olivo y su posterior distribución (Vavilov, 1949-1950; Loukas y Krimbas, 1983; Zohary y Hopf, 1994), siendo este un tema muy controvertido. Para intentar aclararlo, es necesario estudiar las relaciones

genéticas entre las distintas variedades de olivo.

Por último, uno de los obstáculos en la obtención de nuevas variedades de olivo es el gran desconocimiento que se tiene del control genético de las características agronómicas, de especial relevancia en el desarrollo de mapas de ligamiento y de QTLs en olivo. Estos últimos podrían ser de gran provecho para poder identificar asociaciones de interés para la selección temprana de variedades nuevas de olivo, mediante la mejora asistida por marcadores (MAS).

II.2. Objetivos

La hipótesis anterior justifica los objetivos quedando resumidos en los siguientes puntos:

1. Utilización de los microsatélites y SNPs disponibles en olivo para la caracterización e identificación de variedades de olivo argelinas (Characterization and identification of the main algerian olive cultivars by molecular markers. Journal of Horticultural Science and Biotechnology. 2012. 87 (2); 95-100).

2. Desarrollo de nuevos marcadores DArT en el olivo (Development of DArT markers in olive and usefulness of DArT markers in olive variability studies and genome mapping. Scientia Horticulturae, 2012, 136, 50-60).

3. Utilización de los nuevos marcadores DArT para: (i) estudio de la variabilidad de cultivares de olivo procedentes del Banco de Germoplasma Mundial de Olivo de Córdoba y (ii) creación de un mapa de ligamiento junto con SSRs (Development of DArT markers in olive and usefulness of DArT markers in olive variability studies and genome mapping. Scientia Horticulturae. 2012, 136, 50-60).

4. Utilización de los nuevos marcadores DArT, caracteres agronómicos, microsatélites y marcadores SNP, para crear una colección nuclear, representativa de toda la variabilidad que podemos encontrar en el Banco de Germoplasma Mundial de Olivo de Córdoba (Developing a core collection of olive (*Olea europaea* L.) based on molecular markers (DarTs, SSRs, SNPs) and agronomic traits. Tree Genetic and Genomes. 2012. 8, 365-378).

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CAPÍTULO III

Characterisation and identification of olive cultivars from North-eastern Algeria using molecular markers

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Characterisation and identification of olive cultivars from North-eastern Algeria using molecular markers

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III.1. SUMMARY

In the present study, ten microsatellite (simple sequence repeat; SSR) and seven single nucleotide polymorphism (SNP) markers were used to characterise a set of 14 cultivars of olive (*Olea europaea* L.) from North-eastern Algeria. The genetic profiles of these cultivars were compared with two Tunisian, one Moroccan, and two Algerian cultivars. A total of 77 alleles were amplified for the SSR markers. The mean values of the expected heterozygosity (H_e), the observed heterozygosity (H_o), and the polymorphism information content (PIC) values were 0.806, 0.851, and 0.752, respectively. A combination of three SSR loci (ssrOeUA-DCA9, ssrOeUA-DCA18, and UDO099-043) permitted the identification of all 14 olive accessions analysed. Although less polymorphic than SSR markers, the SNP markers used in this study showed relatively high diversity values. The mean H_e value was 0.374. The same value was obtained for the H_o, and PIC values ranged from 0.373 (Cycl-1) to 0.117 (LS2). Eleven out of all 19 olive cultivars (63%) were identified by means of which SNP markers, confirming the value of differences with SSR markers in terms of their efficiency for cultivar discrimination and stressing the need to develop more SNP markers. The results obtained in this work highlight the rich genetic diversity available in even a small set of olive germplasm from Algeria and confirmed the utility of SSR and SNP markers for genotyping olive cultivars.

III.2. Introduction

Olive tree (*Olea europaea L.)* cultivation has evolved around the Mediterranean basin, producing enormous genetic diversity. In fact, approx. 1,200 different olive cultivars have been described in the major olive oil producing countries (Bartolini *et al*., 2005). The cross-pollinating nature of this species and its long

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history have contributed to a rich olive germplasm. This genetic diversity, however, has often resulted in some disadvantages in tree management and varietal identification. The names of many cultivars further confuse their identity, mainly referring to specific morphological traits of the fruit, to the site of cultivation, or to their practical use. This confusion has led to the presence of many cases of homonymy and synonymy in olives.

Algeria, as for most Mediterranean countries, has an ancient history of olive growing, and the crop has largely been managed by traditional farming systems (Maffre, 1847; Cavaillés, 1938; Loussert and Brousse, 1978). Currently, olive cultivation accounts for 33% of the national fruit tree growing area and 2% of arable land use, with continued expansion of the olive growing area predicted. Olive cultivation is situated mainly in the northern part of the country, where most olive orchards (80%) are located in mountainous areas with poor soils (Ministry of Agriculture, 2009).

Algerian olive cultivars are very diverse, and most are represented by old trees that are cultivated locally and their naming carried out by traditional criteria (Hauville, 1953; Mendil and Sebai, 2006). For instance, the names of Algerian olive cultivars may reflect their agronomic and/or morphological characteristics, such as ripening time (e.g., ‘Chetoui’: late winter ripening), and their possible site of origin (e.g., ‘Chemlal de Kabylie’). Hauville (1953) reported the presence of 150 olive cultivars in the country. In a recent survey based on morphological and agronomic data, only 36 cultivars were recognised (Mendil and Sebai, 2006). These contrasting reports suggest the need to deepen our genetic knowledge on Algerian olive patrimony and to quantify the possible risks of loosing genetic diversity. The use of molecular markers could help clarify the range of olive genetic resources in Algeria.

To our knowledge, studies on the genetic diversity and identification of Algerian olive cultivars using nuclear DNA markers have not been conducted so far. Few cultivars from Algeria have been included in previous studies at the Mediterranean level (Besnard *et al*., 2001; Belaj *et al*., 2002; Idrissi and Ouazzani, 2003; Sarri *et al*., 2006), and only a few cultivars from Algeria have

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been included in previous mitochondrial and chloroplast DNA polymorphism analyses (Besnard *et al*., 2001; Besnard and Bervillé 2002; Mariotti *et al*., 2010).

Single sequence repeat (SSR) markers have become the markers of choice for identification and diversity studies of olive cultivar (Belaj *et al*., 2004b; Sarri *et al*., 2006; Baldoni *et al*., 2009); currently, few studies on the characterisation and identification of olive cultivars using single nucleotide polymorphism (SNP) markers have been performed (Reale *et al*., 2006).

In the present study, SNP and SSR markers were used to characterise and identify 14 olive cultivars from North-eastern Algeria, which is an important region in terms of olive cultivation and where most of the representative cultivars of the country are cultivated.

III.3. Materials and methods

III.3.1. Plant material and DNA isolation

Molecular analyses were performed on 19 olive cultivars (Table III.1), 16 from Algeria and three from nearly Morocco (‘Picholine Marocaine’) and Tunisia (‘Chemlali’ and ‘Chetoui’). Fourteen cultivars were collected in North-eastern Algeria and were kindly provided by the University of Skikda, while five cultivars came from the World Olive Germplasm Bank of Cordoba, IFAPA “Alameda del Obispo”, Spain. The cultivars, ‘Chemlal’, ‘Azeradj’, and ‘Bouricha’ occupy 40%, 10% and 5% of the olive orchards in Algeria, respectively. The other cultivars had local distributions in the Skikda Province.

Total DNA was extracted from 0.25g of fresh leaves according to the protocol previously described by De la Rosa *et al*. (2002).

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Tab III. 1. Cultivar names, country of origin and sample provenance

Cultivars	Country of origin	Source of plant material
'Azeradj'	Algeria	SKIKDA*
'Balbal'	Algeria	SKIKDA
'Blilti'	Algeria	SKIKDA
'Bouricha'	Algeria	SKIKDA
'Braouki'	Algeria	SKIKDA
'Chemlal de Kabilye'	Algeria	WOGB (118)
'Chemlal'	Algeria	SKIKDA
'Chetoui-A'	Algeria	SKIKDA
'Derdouri'	Algeria	SKIKDA
'El-Kharfi'	Algeria	SKIKDA
'Kerdoussi'	Algeria	SKIKDA
'Laaninbi'	Algeria	SKIKDA
'Lahmar'	Algeria	SKIKDA
'Lokchiri'	Algeria	SKIKDA
'Rouihni'	Algeria	SKIKDA
'Sigoise'	Algeria	WOGB (119)
'Chemlali'	Tunisia	WOGB (744)
'Chetoui –T'	Tunisia	WOGB (113)
'Picholine Marocaine'	Morocco	WOGB (101)

SKIKDA: Skikda University, Faculté des Sciences et des Sciences de l'Ingeniorat, Université du 20 Aout 1955. Skikda, Algeria.
WOGB: World Olive Germplasm of Cordoba, IFAPA, Spain. (Registration number)

III.3.2. SSR and SNP markers.

Ten SSR and seven SNP markers were tested on all 19 olive cultivars (Table III.1). The SSR markers, ssrOeUA-DCA3, ssrOeUA-DCA5, ssrOeUA-DCA9, ssrOeUA-DCA11, and ssrOeUA-DCA18 were identified by Sefc *et al*. (2000). GAPU59, GAPU71B and GAPU103A had been developed by Carriero *et al*. (2002); while EMO3 and UDO99-043 were described by De la Rosa *et al*. (2002) and Cipriani *et al*. (2002), respectively. Most of these SSR markers have been previously proposed as the most appropriate for DNA fingerprinting studies (Baldoni *et al*., 2009).

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PCR amplification reactions (20 □l) were carried out as described by De la Rosa *et al*. (2002). The PCR products were separated using an automatic capillary sequencer (ABI 3130 Genetic Analyzer; Applied Biosystems/HITACHI, Foster City, CA, USA) at the Unit of Genomics of the Central Service for Research Support of the University of Cordoba, Spain. Genescan Version 3.7 and Genotyper 3.7 software from Applied Biosystems were used for sample analysis and the determination of peak site.

All the SNP markers (Cycl-1, Cycl-2, Lup, Ant-1, LS5, Cbp, and LS2) have been described by Reale et al. (2006). PCR amplifications were performed following the conditions described by these authors. The PCR products were loaded on an ABI Prism 3100 (Applied Biosystems) using the POP-4 separation polymer under standard running conditions. The software was the same as that used for SSR analyses.

III.3.3. Data analysis.

The polymorphism information content (PIC), null alleles, the expected and observed heterozygosities (H_e and H_o) of the SSR and SNP markers were estimated using CERVUS software Version 3.0.3 (Marshall *et al*., 1998).

For each SNP locus, the most frequent allele was designated as the major allele, while the least common one was characterised as the minor allele. Data were scored as present (1) or absent (0) for the major or the minor alleles (homozygous) or the presence of both alleles (heterozygous) for each sample.

III.3.4. Analysis of genetic relationships

The polymorphic profiles were used to create a matrix where alleles were scored as being present (1) or absent (0). Each matrix was generated using the NTSysPc programe Version 2.02 (Rohlf, 1998). Genetic similarities among cultivars were estimated using Dice coefficients (Dice, 1945) and were used for cluster analysis using the UPGMA algorithm.

III.4. Results

III.4.1. SSR and SNP polymorphism and discrimination capacity

The SSRs used showed a high level of polymorphism and discriminating power. The ten SSRs used in this study revealed a total of 77 alleles (Table III.2). The mean values of H_e and H_o were 0.806 and 0.851, respectively. While the mean PIC value was 0.752. Null alleles were found for three SSR loci (ssrOeUA-DCA3, EMO3, and GAPU103 A). The combination of the allelic patterns of three loci (ssrOeUA-DCA9, ssrOeUA-DCA18, and UDO099-043) was able to distinguish all 19 accessions analysed.

The Algerian cultivar ‘Sigoise’ and the Moroccan cultivar ‘Picholine Marocaine’ only showed differences at one (UDOO99-043) out of the ten SSR loci analysed (data not shown).

The SNP markers used showed lower levels of polymorphism and discriminating power than the SSRs (Table III.2). The H_e and H_o values were both 0.374. PIC values ranged from 0.117 (LS2) to 0.373 (Cycl-1). The seven SNPs were not sufficient to identify each of the 19 cultivars under study.

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Tab III.2. Polymorphism revealed by ten SSR and seven SNP markers 19 olive cultivars.

Marker Type	Name	Number of alleles	H_o	H_e	PIC	R
SSRs	ssrOeUA-DCA3	7	0.684	0.801	0.752	0.047
	ssrOeUA-DCA5	6	0.789	0.710	0.659	-0.068
	ssrOeUA-DCA9	11	0.941	0.893	0.853	-0.046
	ssrOeUA-DCA11	7	1	0.783	0.719	-0.171
	ssrOeUA-DCA18	10	1	0.890	0.852	-0.076
	UDO99-043	9	0.824	0.840	0.790	-0.010
	GAPU59	8	0.867	0.722	0.659	-0.121
	GAPU71B	5	0.867	0.733	0.658	-0.098
	GAPU103 A	7	0.786	0.860	0.806	0.028
	EMO3	7	0.750	0.833	0.773	0.034
Average		7.7	0.851	0.806	0.752	-0.020
Max		11	1	0.893	0.853	0.047
Min		5	0.684	0.710	0.658	-0.171
SNPs	Cbp	2	0.235	0.214	0.186	-
	Lup	2	0.438	0.466	0.349	-
	LS5	2	0.263	0.491	0.364	-
	Ant-1	2	0.235	0.299	0.248	-
	LS2	2	0	0.129	0.117	-
	Cycl-1	2	0.727	0.519	0.373	-
	Cycl-2	2	0.533	0.497	0.365	-
Average		2	0.347	0.373	0.286	-
Max		2	0.727	0.519	0.373	-
Min		2	0	0.129	0.117	-

The number of alleles, the observed (H_o) and expected heterozygosity (H_e), the polymorphic information content (PIC) and the frequency of null alleles (R) are reported for each locus.

III.4.2. Genetic relationships among olive cultivars

A dendrogram based on the SSR marker data (Figure III.1A) showed that the average similarity value among genotypes (similarity matrix not shown) was 0.36. The cophenetic correlation coefficient between the dendrogram and the original distance matrix was not high but it was significant (r = 0.74).

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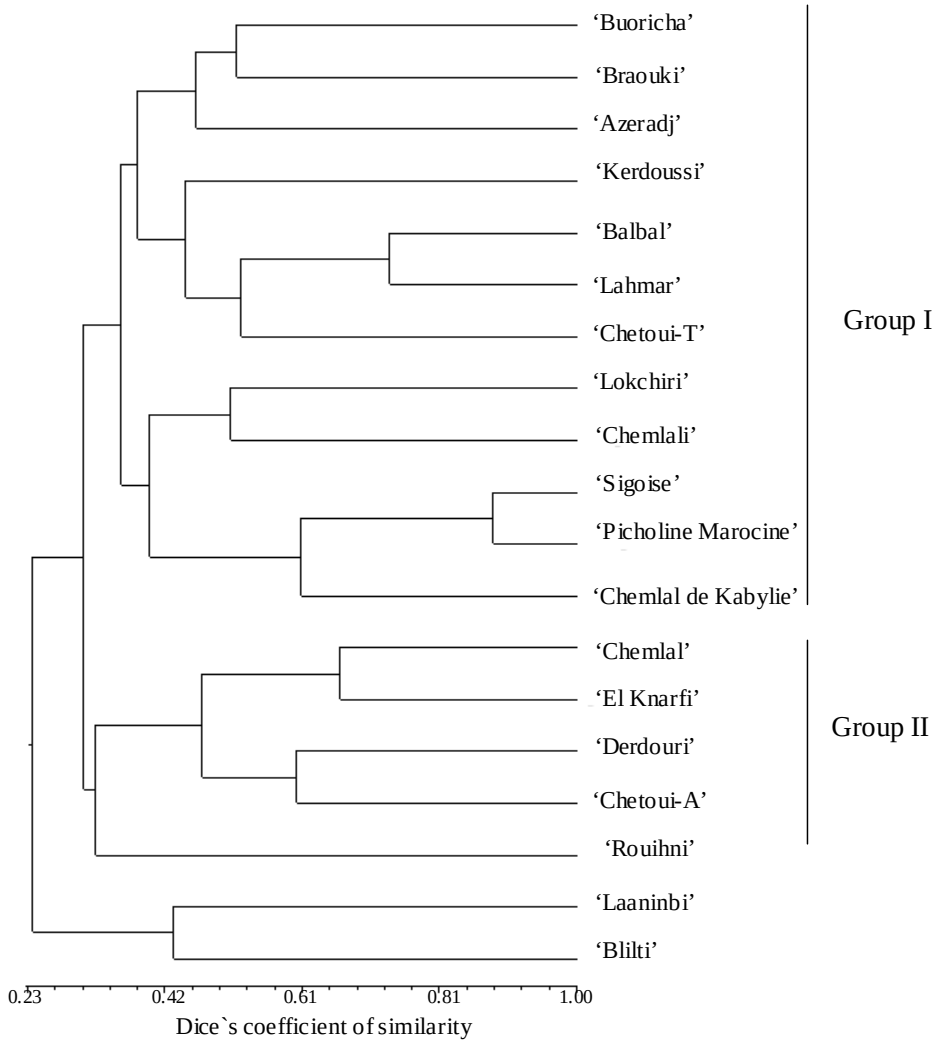
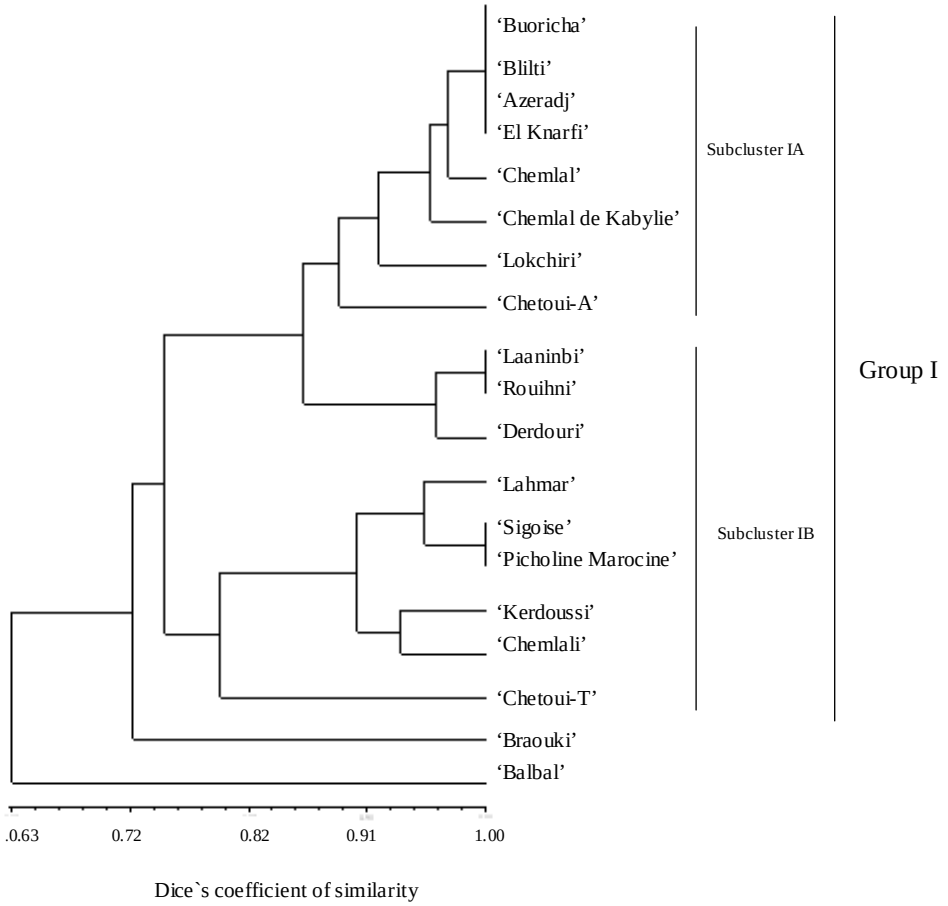
From the dendrogram, most cultivars could be classified into two main Groups. The first Group included nine Algerian, two Tunisian and one Moroccan cultivars. Interestingly, ‘Sigoise’ from Algeria and ‘Picholine Marocaine’ from Morocco clustered together in this group at a high similarity value (0.88). The second main gGroup included only cultivars from North-eastern Algeria (‘Chemlal’, ‘El Knarfi’, ‘Derdouri’, ‘Chetoui-A’, and ‘Rouihi’). The two remaining cultivars ‘Laaninbi’ and ‘Blilti’ clustered separately from the rest of the cultivars.

Fig III.1. Dendograms of 19 olive cultivars using the UPGMA clustering method. Dendrogram of olive cultivars based on SSR markers (Panel A). T; Tunisian germplasm Bank, A; North-eastern Algeria.



The dendrogram based on SNP data (Figure III.1B) showed that the average similarity value among genotypes was 0.75. The cophenetic correlation coefficient between the dendrogram and the original distance matrix was not very high but was significant (r = 0.67), and in the dendrogram, most of the cultivars separated into one group with two subclusters. The first one only had cultivars from North-eastern of Algerian and the second one had cultivars from others area of Algeria and other countries. Additionally, only the two cultivars 'Braouki' and 'Balbal' clustered separately. The cultivars 'Bouricha', 'Blilti', 'Azeradj', and 'El Knarfi' were indistinguishable, including the pairs of cultivars 'Rouihini' and 'Laaninbi' or 'Sigoise' and 'Picholine Marocaine', because SNPs had a lower discriminatory capacity than SSR markers.

Fig III.1. (Continued) Dendograms of 19 olive cultivars using the UPGMA clustering method. Dendrogram of olive cultivars based on SSR markers (Panel B). T; Tunisian germplasm Bank, A; North-eastern Algeria.



The average similarity value among genotypes based on both SSR and SNP markers data was 0.68, with most similarity measures among genotypes ranging between 0.36 and 0.90. In this case, the cophenetic correlation coefficient between the dendrogram and the original distance matrix was not very high but significant (r = 0.74). In the consensus dendrogram (Figure III.1C), most of cultivars could be classified into two main groups. The first group was very heterogeneous, with varieties common to all three dendrograms based on SSRs, SNPs, and both markers. Those varieties were ‘Rouihni’, ‘Bouricha’, ‘Chemlal de Kabylie’, ‘Sigoise’, ‘Picholine Marocaine’, and ‘Kerdoussi’. The cultivars ‘Sigoise’ from Algeria and the cultivar ‘Picholine Marocaine’ from Morocco clustered together in all dendrograms, with a mean value of similarity of 0.90. The rest of cultivars (‘Blilti’ and ‘Azeradj’) were from North-eastern Algeria, and they clustered separately from the rest of cultivars.

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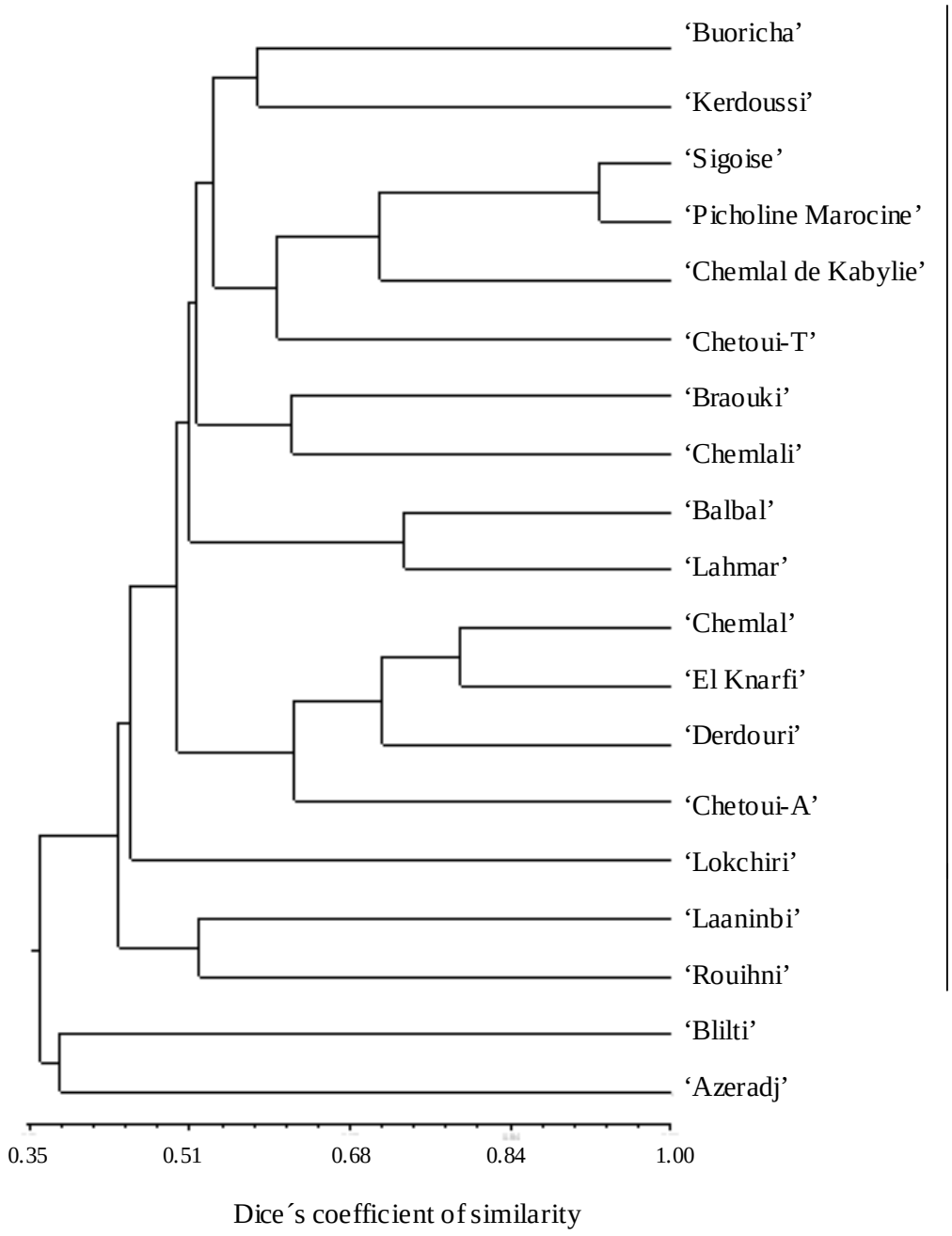
Fig III.1. (Continued) Dendograms of 19 olive cultivars using the UPGMA clustering method. Consensus dendogram of olive cultivars based on both SSR and SNP markers (Panel C). T; Tunisian germplasm Bank, A; North-eastern Algeria.



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III.5. Discussion

III.5.1. Genetic polymorphism assessed by SSR and SNP markers

The results obtained in this work confirmed the high efficiency of SSR and SNP markers for genotyping olive cultivars and highlighted the genetic richness available in a small set of the Algerian olive germplasm, as previously shown by morphological descriptors (Barranco et al., 2000; Mendil and Sebai, 2006). Unfortunately, differences in morphological traits were observed between the two morphological studies of the same cultivar. As a result, the fruit shape of cultivar 'Azeradj' was considered ovoid by Barranco et al. (2000) and flattered by Mendil and Sebai (2006). Similar differences were found for the same cultivar for the position of maximum diameter, which was described as central by Barranco et al. (2000) and toward the base by Mendil and Sebai (2006), including the stone surface, rough (Barranco et al., 2000) and smooth (Mendil and Sebai 2006). Other cases of morphological differences have been reported by the same authors regarding the cultivars 'Sigoise' and the Tunisian 'Chemlali', and these findings have revealed that the use of only one type of descriptor can be misleading, while the combined use of molecular markers and morphological characterisation could guarantee the identity of olive cultivars (Belaj et al., 2011).

The results of the SSR loci included in this analysis were highly polymorphic as previously reported (Baldoni *et al.*, 2009). The number of alleles obtained was similar to that reported by Poljuha *et al*. (2008) and Bracci *et al*. (2009) but somewhat lower than that published by Alba *et al.* (2009), probably because this study includes a low number of cultivars and a different set of SSR loci. Additionally, the expected heterozygosity found in the full set of cultivars was similar to that reported from several authors in studies about germplasm from other olive growing areas (Bandelj *et al*., 2002; Belaj *et al*., 2004b; Reale *et al*., 2006). These results are also consistent with those reported in several studies performed by SSR markers on the olive germplasm of other North African countries (Charafi *et al*., 2008; Rekik *et al*., 2008; Taamalli *et al*., 2008).

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The SNP markers showed a lower level of polymorphism than SSRs, which was due to these markers representing single nucleotide mutations, normally of a bi-allelic nature (Varshney *et al.*, 2007). Previous studies (Laval *et al*., 2002) indicated that to achieve a genetic variability similar to a set of SSRs with k alleles, then k - 1 times more biallelic markers (as SNPs) are needed. It has been shown that ten or more SNPs hold information content equal to just one SSR in terms of the number of alleles detected (Yan *et al*., 2009).

The average H_e of 37% for the 7 SNPs assayed in the 19 olive cultivars was lower than that published by Reale *et al*. (2006), probably because of the differences in the plant material used.

The high PIC values found for SSRs are consistent with previous reports (Belaj *et al*., 2004b; Khadari *et al*., 2008; Poljuha *et al*., 2008). The lower PIC values found for SNPs could be explained by their biallelic nature, calling for the extension of the analysis to a higher number of SNP loci. In fact, one of our objectives is the identification of new SNP markers for the olive varietal genotyping. In addition to microsatellites, these markers are useful tools for the exchange of data between laboratories (Varshney *et al*., 2007).

III.5.2. Utility of SSRs and SNPs for olive cultivar identification and genetic relationships

Only three SSRs were sufficient to identify the full set of cultivars under study, while SNPs were less informative.

A high level of polymorphism has been found between accessions carrying the same name. In the case of ‘Chemlal’, the differences between the two accessions included in the study are so wide that they certainly represent different cultivars. Previous studies conducted with morphological (Lombardo *et al*., 2004) or molecular markers (Besnard *et al*., 2001; Belaj *et al*., 2004a,b; Rekik *et al*., 2008; Muzzalupo *et al*., 2009a, b) have demonstrated that cultivars under the same name may be included in different genotypes. Moreover,

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differences among ‘Chemlal’ accessions have also been found via chloroplast and mitochondrial marker analyses (Besnard *et al*., 2000) as well as by AFLPs (Grati Kamoun *et al*., 2006). The same could be said for the two ‘Chetoui’ accessions, as the two genotypes assayed displayed very different results as previously reported (Taamalli *et al*., 2007).

Interestingly, the cultivars ‘Sigoise’ and ‘Picholine Marocaine’ from Algeria and Morocco, respectively, always clustered together. Only one SSR (UDOO99-043) was able to discriminate between these cultivars. The studies published by Barranco *et al.* (2000) performed with morphological descriptors indicated that they are similar. However, previous studies using RAPD (Belaj et al., 2002), SSR (Sarri *et al.*, 2006) and morphological (Idrissi and Ouazzani, 2006) markers have evidenced the presence of important differences between these two cultivars. Also, studies comparing their chloroplasts (Mariotti, pers. comm.) reveal the same conclusion.

The three dendrograms obtained from SSRs, SNPs and both markers showed differences on the clustering of the cultivars, resulting from the different type and level of variability between the two types of markers. However, the varieties ‘Bouricha’, ‘Chemlal de Kabylie’, ‘Sigoise’, ‘Picholine Marocine’ and ‘Kerdoussi’ clustered together in all three dendrograms.

In conclusion, both SSR and SNP markers appear suitable to explore the genetic variability of the Algerian olive cultivars, even though SNP markers showed a lower discrimination capacity compared to SSR markers. The cases of homonymy found indicate the necessity of a systematic prospection and molecular characterisation of cultivars in order to sort out the rich olive germplasm of this country. Despite the low number of cultivars studied, our results suggest the existence of great variability in the Algerian olive germplasm, which has been scarcely explored. This variability needs to be further evaluated and conserved both *in situ* and into germplasm banks. In this way, Algerian growers and breeders may rely in the future on their autochthonous cultivars for local selection and breeding.

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CAPÍTULO IV

Development of DArT markers in olive (*Olea europaea* L.) and usefulness in variability studies and genome mapping

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Development of DArT markers in olive (*Olea europaea* L.) and usefulness in variability studies and genome mapping

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IV.1. Abstract

The present study reports the setting up of Diversity Arrays Technology (DArT) markers in olive (*Olea europaea* L.). Two genomic representations were generated using the *Pst*I/*Taq*I combination. A first one was aimed to cover the variability available at the World Olive Germplasm Bank (WOGB) and it was based on DNA from 87 olive cultivars carefully selected from the WOGB to represent the olive variability. The second one was obtained from DNA of the parents of a mapping population, 'Picual' and 'Arbequina', and was used to increase the number of markers segregating within them. A total of 2031 and 1630 markers were used for diversity and mapping analyses, respectively to test the utility of DArT markers in olive. A set of 62 cultivars was genotyped with olive-specific DArT markers. A dendrogram was constructed confirming the relationships among olive cultivars reported in previous works. Besides, DArT markers permitted the unambiguous discrimination of all 62 genotypes confirming thus their utility for identification studies, an important issue for management of germplasm collections. DArT markers also allowed the construction of a genetic map of olive using the population derived from the cross 'Picual' x 'Arbequina' and the pseudo-testcross mapping strategy. This map will be used as a framework map to add new markers derived from olive sequencing originating from the project OLEAGEN. In conclusion, olive-specific DArT markers will enhance identification and genetic studies in olive given their high throughput and low cost per data.

Keywords: DArTs, *Olea europaea,* linkage map, genetic variability.

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IV.2. Introduction

Olive tree (*Olea europaea)* is one of the oldest fruit tree species cultivated in Mediterranean Basin with an important economical value. More than 1,200 varieties have been so far described (Bartolini et al., 2005), most of them come from the empirical selection of the growers and are still restricted to their area of origin (Besnard et al., 2001). Olive has relatively small (2200 Mb) diploid (2n=46) genome, predominantly allogamous nature and it is also characterized by its wide genetic patrimony, represented of thousands of local and old cultivars (Bartolini et al., 2005; De la Rosa et al., 2003). This huge genetic variability has been evaluated by different molecular markers including RAPDs (Durgac et al., 2010; Hagidimitriou et al., 2005), AFLPs (Rao et al., 2009), RFLPs (Besnard and Bervillé, 2002; De Caraffa et al., 2002), SCARs (Shu-Biao et al., 2004), ISSRs (Hess et al., 2000; Vargas and Kadereit, 2001), SSRs (Díaz et al., 2006; Sarri et al., 2006), and SNPs (Reale et al., 2006; Rekik et al., 2010). These works have allowed for cultivar identification and for diversity studies. However, the gap between the available diversity and its effective use is still very large (Belaj et al., 2011a). Despite of the advance in molecular markers the knowledge on the genetic basis of traits of interest in olive is scarce. To our knowledge, few genetic maps have been published (Baldoni et al., 1999; De la Rosa et al., 2003; El Aabidine et al., 2010; Khadari et al., 2010; Wu et al., 2004). Furthermore, the genetic control of the most important traits in olive is still unknown. Indeed, no QTL has been identified yet in olive.

In many important forest and fruit trees, the time required to reach the adult stages supposes a serious limitation for QTL mapping. Thus, linkage disequilibrium mapping offers new opportunities and enables fast trait improvement (Ersoz et al., 2007). In either case, the lack of high throughput molecular markers systems with low cost per data constitutes a significant barrier for olive breeding. Although some attempts have been made to release new cultivars such as ‘Chiquitita’, the development of early selection techniques such as markers assisted selection could speed up the generation of new olive cultivars (Rallo et al., 2008). Although sequencing activities within the project OLEAGEN are providing very useful data

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(https://chirimoyo.ac.uma.es/oleagen), the development of high-throughput marker systems based on the sequence information will not be available in the near future. Meanwhile, there is an increasing need of low cost, high-throughput markers allowing for easy analysis and good genome coverage to allow genetic studies including QTL detection and LD mapping in olive.

Diversity Arrays Technology (DArT) markers were developed in rice (Jaccoud et al., 2001) and they allow whole-genome profiling of >60 organisms (www.diversityarrays.com) without the need for sequence information. Thus, DArT markers offer an interesting alternative for olive. Indeed, this technology has been successfully applied to different species with low information at the sequence level such as oat (Tinker et al., 2009), *Musa* (Risterucci et al*.,* 2009), rye (Bolibok-Bragoszewska et al., 2009), *Sorghum* (Mace et al., 2008) or even wild species such as *Hordeum chilense* (Rodriguez-Suárez et al., in press). In woody species, DArT technology has only been used in banana (Amorim et al., 2009; Hippolyte et al., 2010; Risterucci et al., 2009), cassava (Hurtado et al., 2008; Xia et al., 2005), eucalyptus (Sansaloni et al., 2010) and apple (Schouten et al., 2011) for genetic variability and mapping studies.

The objective of this study is to report the development of Diversity Arrays Technology (DArT), as the first high throughput marker system developed on olive. Its utility for assessing the genetic diversity in a set of cultivars and to construct a linkage map is also evaluated.

IV.3. Material and Methods

IV.3.1. Plant material and DNA extraction

Eighty-seven olive cultivars (Table IV.1). were selected from the World Olive Germplasm Bank (WOBG). This set includes cultivars from 16 different countries and most of them had been previously characterized for morphological traits (Barranco and Rallo, 1984), RAPDs (Belaj et al., 2001), microsatellite markers (Rallo et al., 2000; Diaz et al., 2006) and SNPs (Reale et al., 2006, Rekik et al., 2010).

Additionally, 91 seedlings derived from the cross 'Picual' × 'Arbequina' from the cooperative breeding program of the University of Córdoba and IFAPA, Spain were also included in the study as mapping population.

Total genomic DNA was extracted from fresh leaves according to the protocol previously established for Murray and Thompson (1980) with the modifications described in De la Rosa et al. (2002) and using Guanidine Thiocyanate for protein precipitation.

IV.3.2. Microarray preparation

DNA samples were processed as described in detail by Heller-Uszynska et al. (2010). Briefly, ~100 ng of DNA of each sample was used. Digestion and ligation reactions were carried out simultaneously with 2 units of *PstI* and *TaqI* restriction enzymes (NEB), 80 units of T4 DNA ligase (NEB) and 0.05 µM of adaptors (5'-CACGATGGATCCAGTGCA-3' and 5'-CTGGATCCATCGTGCA-3'). One microliter of the digestion/ligation product was used as template for amplification with 2 units of *RedTaq* DNA polymerase (Sigma) and 0.4 µM of primer (5'-GATGGATCCAGTGCAG-3'). The PCR program was as follows: 72ºC for 7 min, 95ºC for 3 min, 30 cycles of 94ºC for 20 s, 58ºC for 40 s, 72ºC for 1 min followed by 72ºC for 7 min.

Two pools of the amplified genomic representations were assembled: the first pool with diversity samples listed in Table IV.1 to generate the diversity library and array, and the second one with 'Picual' and 'Arbequina' to construct the mapping library and array. The pools were ligated separately into the PCR2.1-TOPO vector using a T/A cloning kit (Invitrogen, Carlsbad, CA, USA), then transformed into electrocompetent *Escherichia coli* strain TOP10F´ (Invitrogen) according to the manufacturer's instructions. Transformants were selected on a medium containing 100 µg/mL amplicillin and 40 µg/mL X-gal. Individual transformant colonies were transferred into 384 well plates filled with LB containing 100 µg/mL ampicillin and mix of salts (Jaccoud et al., 2001) facilitating subsequent PCR amplification directly from the medium (unpublished observation). A 0.5 µl aliquot of bacterial culture was used as a template for

insert amplification with 0.2 µM of each M13 Forward and M13 Reverse primers (Invitrogen). The cycling conditions were as follows: 95℃ for 4 min, 57℃ for 35s, 72℃ for 1 min followed by 35 cycles of 94℃ f or 35s, 52℃ for 35s and 72℃ for 1 min and final 72℃ for 7 min. The purifi ed amplicons were suspended in the spotting buffer and printed in duplicate onto the poly-$_L$-lysine coated microarray slides (Erie Scientific) using a *MicroGridII* arrayer (Biorobotics). After printing the DNA deposited onto slides was denatured and fixed to the surface of the microarray slide.

Tab IV.1. Olive genotypes used for DArT representation, selected in Word Olive Germplasm Bank of Cordoba.

IG number	Row-tree	Cultivar	Origin
840	**45-11**	'Abou Kanani'	Syria
723	**31-23**	'Aggezi Shami-1'	Egypt
692	**10-15**	'Agouromanakolia'	Greece
696	**1-16**	'Amigdalolia Nana'	Greece
231	**4-7**	'Arbequina'	Spain
666	**29-22**	'Arbosana'	Spain
1068	**47-12**	'Argudell'	Spain
62	**10-5**	'Ascolana Tenera'	Italy
726	**24-9**	'Azapa o Arauco'	Chile
1026	**50-5**	'Barri'	Syria
48	**16-2**	'Blanqueta-48'	Spain
18	**18-12**	'Bolvino'	Spain
41	**22-13**	'Borriolenca'	Spain
63	**16-5**	'Bouteillan'	France
653	**20-17**	'Canetera'	Spain
77	**21-12**	'Caninese'	Italy
332	**31-11**	'Carrasqueño de Alcaudete'	Spain
286	**17-11**	'Carrasqueño de la Sierra'	Spain
220	**3-6**	'Chalkidikis'	Greece
118	**6-5**	'Chemlal de Kabilye'	Algeria
402	**34-17**	'Chorreao de Montefrio'	Spain
90	**20-10**	'Cipresino'	Italy
79	**31-20**	'Coratina'	Italy
645	**23-17**	'Corbella'	Spain
10	**24-4**	'Cornicabra'	Spain

Table IV.1.(Continued) Olive genotypes used for DArT representation, selected in Word Olive Germplasm Bank of Cordoba.

IG number	Row-Tree	Cultivar	Origin
1072	**47-7**	'Curivell'	Spain
539	**26-17**	'Dokkar'	Turkey
94	**30-13**	'Domat'	Turkey
686	**1-11**	'Elmacik'	Turkey
13	**4-6**	'Empeltre'	Spain
128	**5-5**	'Galega Vulgar'	Portugal
735	**1-2**	'Istarska Bjelica'	Croatia
23	**16-13**	'Jaropo'	Spain
667	**20-16**	'Joanenca'	Spain
105	**5-6**	'Kalamon'	Greece
691	**36-14**	'Kalokerida'	Greece
688	**1-4**	'Kan Celebi'	Turkey
1081	**48-10**	'Klon-14-1'	Albania
218	**8-13**	'KoroneikiI'	Greece
704	**17-15**	'Lastovka'	Croatia
5	**29-10**	'Lechín de Sevilla'	Spain
384	**34-15**	'Lentisca'	Spain
703	**17-16**	'Levantinka'	Croatia
226	**17-8**	'Llumeta'	Spain
1462	**3-11**	'Loaime-1'	Spain
430	**21-16**	'M. Cacereña'	Spain
1010	**49-12**	'Mahati-1010'	Syria
152	**30-10**	'Majhol-152'	Syria
760	**12-16**	'Manzanilla de Guadix-1'	Spain
757	**14-15**	'Manzanillera de Huercal Overa'	Spain
84	**17-6**	'Maurino'	Italy
699	**33-10**	'Maureya'	Greece
1013	**52-4**	'Mechjul 1013'	Syria
108	**7-7**	'Megaritiki'	Greece
669	**45-1**	'Menya-669'	Spain
120	**26-13**	'Merhavia'	Israel
864	**44-3**	'Mollar Basto'	Spain
114	**7-2**	'Ouslati'	Tunisia
661	**22-14**	'Palomar'	Spain
649	**32-10**	'Patronet'	Spain
421	**35-17**	'Pecoso'	Spain
562	**29-16**	'Pequeña de Casas Ibañez'	Spain
1170	**58-3**	'Peranzana'	Italy
70	**5-12**	'Picholine'	France
101	**12-7**	'Picholine Marroqui'	Morocco
9	**19-5**	'Picual'	Spain
3	**22-3**	'Picudo'	Spain
969	**49-3**	'Piñonera'	Spain

Table IV.1.(Continued) Olive genotypes used for DArT representation, selected in Word Olive Germplasm Bank of Cordoba.

IG number	Row-Tree	Cultivar	Origin
227	**5-9**	'Rapasayo'	Spain
665	**19-15**	'Sabatera'	Spain
1041	**49-6**	'Shami-1041'	Syria
767	**12-15**	'Torcio de Cabra'	Spain
1078	**50-3**	'Ulliri i Kuq'	Albania
613	**22-17**	'Vallesa'	Spain
664	**20-14**	'Vaneta'	Spain
660	**47-6**	'Vera'	Spain
887	**53-1**	'Verde Verdéelo'	Portugal
883	**42-3**	'Verdial de Badajoz'	Spain
51	**21-9**	'Verdial de Velez-Malaga-1'	Spain
1065	**48-4**	'Vinyols'	Spain
725	**57-18**	'Wardan'	Egypt
689	**2-7**	'Yun Gelebi'	Turkey
738	**13-14**	'Zaity'	Syria
117	**9-2**	'Zalmati'	Tunisia

IV.3.3. Genotyping of DNA samples and scoring

The genomic representations of the olive genotypes were generated with the same complexity reduction method (*PstI/TaqI*) and used to prepare the libraries spotted on the two arrays (diversity and mapping). Genotyping was carried out using methods published in details elsewhere (Heller-Uszynska et al, 2010). In short, targets purified by isopropanol precipitation were labeled with fluorescent dyes (either cy3 or cy5) using Klenow exo$^-$ fragment of *E. coli* Polymerase I (NEB) and random decamers. Labelled targets were suspended in hybridization buffer containing FAM labelled polylinker of the TOPO vector used for cloning and library construction. FAM labelled polylinker served in a subsequent analysis as a reference of DNA quantity present in each feature of microarray and a denominator for a ratio calculated for hybridization intensities of each cy3 or cy5 labelled targets. The hybridization mixture was distributed across microarrays and hybridized 16 h at 62℃ water bath. Slides were then washed in buffers with increasing stringency, dried and scanned in confocal laser scanner (Tecan LS300, Grödig, Austria). Array images were analyzed, polymorphism identified and scored with DArTsoft 7.4 (Diversity Arrays

Technology P/L, Canberra, Australia). This program analyzed three sets of images produced per microarray, one for each resulting from hybridization of cy3 labelled target, cy5 labelled target, and FAM labelled TOPO vector polylinker. DArTsoft recognized array features using a seeded-region-growth algorithm, extracted the relative hybridization intensity data, identified and scored polymorphism and calculated a range of quality parameters for each marker. The relative hybridization intensity data for all markers across all genotyped samples were binarized into two clusters: present (“1”) vs. absent (“0”) using fuzzy k-means clustering. The relative hybridization intensity data which could not be binarized (low classification probabilities) were rejected and set as no data values. Accordingly, markers were scored “1” for presence, “0” for absence, and “-” for no data. DArTsoft was used to automatically analyze batches of up to 96 slides to identify and score polymorphic markers.

The quality parameters used for selection of polymorphic markers in the presented work were: call Rate (percentage of markers effectively scored), Q value (an ANOVA based quality parameter indicating how well two clusters - present “1” vs. absent “0”- were separated in the set of targets, with high Q values selecting reliable markers), and reproducibility (measure of how reproducible was scoring between replicated targets).

IV.3.4. Diversity analysis

DArT markers obtained were analyzed with DArTSoft and only those having a threshold criteria of reproducibility and call rate higher than 97% and 80%, respectively, were selected. As clones to build an array and library are picked at random, clone redundancy (i.e. DNA fragments with the same or very similar/overlapping sequence) is an issue. This was evaluated with the software package DArT ToolBox (http://www.diversityarrays.com/) by constructing a Hamming distance matrix between clones, followed by distance binning, in which all clones with zero distance were placed into the same bin. Approximately 30% of the potentially redundant markers were maintained, similarly to what has been done in *Eucalyptus* (Sansaloni et al., 2010).

Diversity of DArT markers was analyzed using the following parameters: (a) polymorphism information content (PIC), (b) Shannon's diversity index (Sh) and (c) effective number of alleles (Ne).

The polymorphism information content (PIC) values were calculated for each DArT marker using the formula:

$$PIC = 1 - \sum_{i=1}^{I} p_i^2 - 2\left[\sum_{i=1}^{I-1}\sum_{j=i+1}^{I} p_i^2 p_j^2\right]$$

where p_i and p_j are the frequency of the i^{th} and j^{th} alleles at a locus with I alleles in a population, respectively (Botstein et al., 1980).

Shannon's diversity index (Sh; Lewontin, 1972) was calculated as:

$$Sh = -\sum_{i=1}^{I} p_i \log_2 p_i$$

where p_i is the frequency of the i^{th} allele.

The effective number of alleles (N_E; Berg and Hamrick 1997) was estimated as:

$$N_E = \frac{1}{\sum_{i=1}^{I} p_i^2}$$

where p_i is the frequency of the i^{th} allele.

IV.3.5. Analysis of genetic relationships

A set of 62 cultivars (Table IV.1) from the set of 87 used to develop the diversity array was selected on the basis of their geographic origins, identification status and economic importance within the Mediterranean region. Twenty-five genotypes from the diversity array were excluded from this analysis as they were local from Spain in order to avoid over representation of this country in the dendrogram. The selected DArT markers were tested for their ability to resolve genetic relationships among a set of 62 varieties. Pairwise genetic distances were calculated using Dice's coefficient (Dice, 1945). Cluster analysis was

conducted using UPGMA algorithm as implemented in TREECON for Windows ver. 1.3 b (Van de Peer and De Wachter, 1994). Bootstrap analysis was performed on 1,000 bootstrap samples to test the reliability of branches (Felsenstein ,1985).

IV.3.6. Map construction

Marker scores obtained after hybridizations of the mapping population individuals and the parental lines 'Picual' and 'Arbequina' to the diversity array and the mapping array were combined. Markers with a quality parameter Q greater than 77 and a call rate greater than 80 were considered for mapping. Markers with a quality parameter Q between 70 and 77 were incorporated in a case-by-case basis.

Thirty-eight microsatellites previously reported by Carriero et al. (2002), Cipriani et al. (2002), De la Rosa et al. (2002), Diaz et al. (2006), Sabino-Gil et al. (2006) and Sefc et al. (2000) were also tested in the mapping population. This set of primers included the most polymorphic and reliable SSR markers in olive (Baldoni et al., 2009).

JoinMap 4.0 mapping software (Van Ooijen, 2006) was used for mapping. Each marker was tested against the expected segregation ratio using a chi-square goodness of fit. The markers not showing normal diploid segregation ($P < 0.01$) were excluded from further analysis. Markers were grouped into five distinct segregation types as defined in JoinMap 4.0 for "Cross Pollinator (CP)" (a population resulting from a cross between two heterogeneously heterozygous and homozygous diploid parents, linkage phases originally unknown) type as follows: (1) <nnxnp>, (2) <lmxll>, (3) <hkxhk>, (4) <efxeg> and (5) <abxcd>. DArT markers belonged to the first three classes and their segregation ratios were 1:1 for classes 1 and 2 and 3:1 for class 3 since they were dominant. SSRs were distributed in the five classes and their segregation ratios were 1:1 for classes 1 and 2, 1:2:1 for class 3 and 1:1:1:1 for classes 4 and 5.

IV.3.7. Mapping strategy

The mapping population was analyzed using the "CP" type with a pseudo-testcross mapping strategy as described by Grattapablia and Sederoff (1994). Thus, two separate maps for 'Picual' and 'Arbequina' were developed. In this configuration, only markers present in one parent and absent in the other or *vice versa* are useful for mapping (segregation types <lmxll> for 'Picual' map and <nnxnp> for 'Arbequina' map). The markers <hkxhk> are excluded from the analysis while the <abxcd> and <efxeg> are automatically re-codified by JoinMap into <lmxll> and <nnxnp> types.

Linkage groups were determined using an independence LOD threshold of 4.0. Order of markers within chromosomes was established using the maximum likelihood algorithm of JoinMap 4.0. The Kosambi mapping function was used to convert recombination fractions to cM. The map was drawn using MapChart 2.1 software (Voorrips, 2002).

IV.4. Results

IV.4.1. Scoring of DArT polymorphism

On average, DArT markers showed a reproducibility of 99.8% and a call rate of 96.9%. An initial set of 2,857 markers was available for diversity analyses in a subset of 62 olive cultivars, comprising 2,029 markers derived from the diversity array and 828 from the mapping array. From those, 2,031 markers which meet the quality criteria (PIC values, call rate and P-values) (data not shown) were used for diversity analyses.

The redundancy estimation based on distance binning of the 2031, polymorphic markers resulted in 817 unique bins (Table IV.2). With a limited number of effective scores for calculating the distance matrix for markers and a clear genetic structure in the materials used for initial marker discovery, there was a high likelihood of unique sequences being grouped to a single bin, especially in large bins. We maintained approximately 30% of potentially redundant markers,

with frequency of retention proportional to the bin size, similarly to what have been done in *Eucalyptus* (Sansaloni et al., 2010). Therefore, a total of 1,255 markers, 817 unique and 438 potentially redundant, were included in the diversity analysis (Table IV.2).

Tab IV.2. Results of DArT clone redundancy analysis based on DNA sequencing of clones selected from the sixteen bins that had at least 30 clones per bin, based on Hamming distance of zero (no difference in scoring pattern between markers in the bin).

Bin size	No. of bins	Total number of markers per bins	No. of retained markers per bin (appr. 30%)	Total no. of retained markers per bin
1	817	817	1	817
2	155	310	1	155
3	73	219	1	73
4	46	184	1	46
5	26	130	2	52
6	13	78	2	26
7	7	49	2	14
8	8	64	2	16
9	4	36	3	12
10	4	40	3	12
11	2	22	3	6
12	2	24	4	8
13	1	13	4	4
14	1	14	4	4
15	1	15	5	5
16	1	16	5	5
Total		2031		1255
				61.79 %

The diversity array only provided 574 markers useful for mapping the 'Picual' × 'Arbequina' progeny. On the contrary, the mapping array yielded a total of 1,056 markers segregating in the mapping population and thus, the second genomic representation was more efficient to develop markers useful for mapping.

IV.4.2. Genetic diversity and relationships among olive cultivars

The 1,255 DArTs used in the diversity analysis were more than sufficient to discriminate among the 62 cultivars tested. PIC value ranged from 0.5 to 0.018 with an average value of 0.33. The average effective number of alleles per

locus was 1.595 (Table IV.3), ranging from 2 to 1.033. The Shannon's diversity index used to quantify the genetic diversity present in the olive cultivars, ranged from 0.119 to 1 with an average of 0.755.

Tab IV. 3. Polymorphism of 1255 markers tested in the 62 cultivars of WOGB. (Key: Sh: Shannon's diversity index; N_E: effective number of alleles).

Parameter	Sh	N_E	PIC	P	Call rate
Average	0.755	1.595	0.33	81.52	97.82
StDev	0.229	0.294	-		-
Min	0.119	1.033	0.018	50.57	80.82
Max	1.000	2.000	0.5	96.96	100

As shown in the UPGMA dendrogram (Fig. IV.1), DArT markers were able to detect a high variability among the 62 olive cultivars evaluated with relatively high bootstrap values. The number of polymorphic markers among cultivars ranged from 142, (the pair of cultivars 'Verdial de Badajoz'-'Picholine Marrocaine'), to 584 (the pair of cultivars 'Chami-1041'-'Dokkar'). In this dendrogram, most cultivars were classified into 3 clusters which belong to two main groups: Group I (cluster 01 and 02) and Group II (cluster 03). A certain evidence of relationships for most of the cultivars according to their geographic origin was found.

Group I is constituted mainly by cultivars from Western (Spain, France, Portugal, Marocco) and Central (Italy, Greece, Croatia and Tunisia) Mediterranean zone with some exceptions such as the cultivars 'Chemlal de Kabylie' (Algeria), Merhavia (Israel) and Aggezi Shami 1 (Syria). For instance, cluster 01 includes nine Spanish cultivars, four Greek and Italian ones as well as two cultivars from Portugal, France and Croatia, respectively. The cultivar 'Azapa o Arauco' (Chile) is also included in this cluster. Cluster 02 is constituted by Spanish (6), Greek (3), Italian (2), Tunisian (2) and Croatian (1) cultivars.

The second main group was formed by one cluster (cluster 03). All but two cultivars ('Aguromanokia' and 'Bolvino' from Greece and Spain, respectively) did come from Eastern Mediterranean zone. The majority of Syrian (7) and

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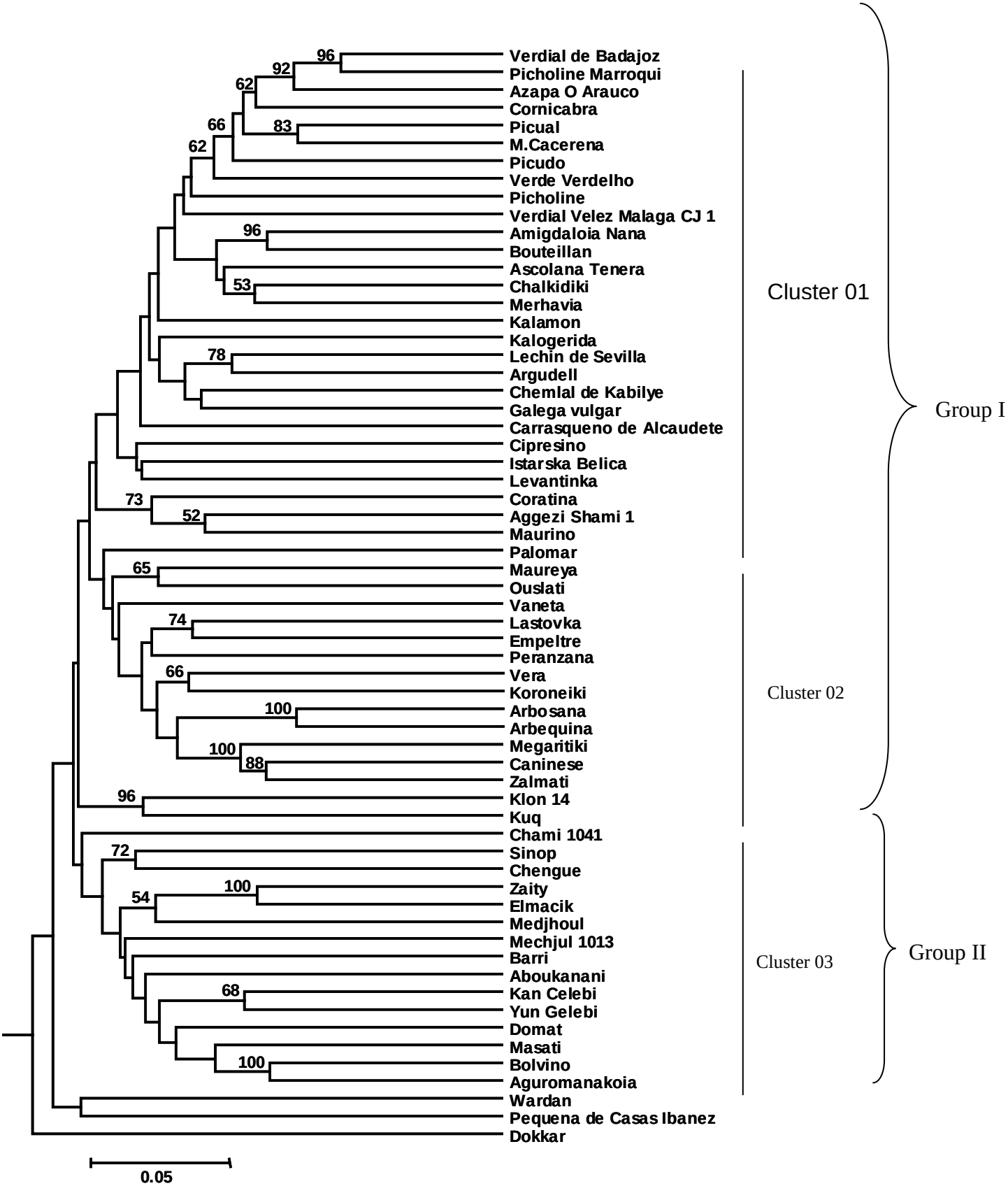
Turkish cultivars (5) as well as the Iranian cultivar ‘Chengue’ did cluster in this group.

Fig IV.1. UPGMA dendrogram based on Dice´s coefficient calculated with 1255 DArT markers. Bootstrap values, based on 1,000 replications, are shown near the corresponding branches.



It is interesting to mention that some pairs of cultivars from the same country did cluster very closely: 'Istarska Bjelica' - 'Levantinka' (Croatia); 'Arbosana'– 'Arbequina' (Spain) and 'Kan Celebi'-'Yun Celebi' (Turkey). Similarly, two Albanian cultivars ('Klon 14' and 'Ulliri i Kuq') did clustered separately in Group I.

Three cultivars 'Wardan' (Egypt), 'Pequeña de Casas Ibanez' (Spain) and 'Dokkar' (Turkey) did cluster separately from the rest of cultivars.

IV.4.3. Map construction and features

A total of 1,630 DArT markers segregated in the mapping population, including diversity markers (574) and mapping markers (1,056). Forty percent of DArTs segregated in 3:1 proportion in the mapping population and thus they are not useful for mapping using the pseudo-testcross strategy. A total of 38 SSRs were tested in our mapping population. JoinMap automatically re-codified these markers to comply with pseudo-testcross mapping. As a result, DArT datasets were supplemented with 29 and 28 SSR loci for 'Picual' and 'Arbequina' maps, respectively. Thus, a total of 422 and 613 markers were available for the construction of 'Picual' and 'Arbequina' maps, respectively. In the present study, a distortion percent of 10% and 24% of the markers used were found on 'Picual' (female parent) and 'Arbequina' (male parent), respectively. Markers showing a segregation distortion at $P < 0.01$ were excluded from mapping since the existence of duplicated loci may result in incorrect scoring leading to segregation distortion. It is not possible to discern between real distorted markers and incorrect scoring without genetic stocks such as chromosome-addition lines and thus we decided to exclude these markers since they may affect the linkage analysis. This resulted in 105 markers excluded from the 'Arbequina' map and 12 markers excluded from 'Picual' map.

The genetic map obtained is shown in the Fig. IV.2. A total of 47 and 39 linkage groups with two or more markers were obtained for 'Picual' and 'Arbequina' maps, respectively. Twenty-three linkage groups are shown for each map (Fig. IV.2). The smallest linkage groups are not shown since they are usually

composed by just two markers. We exclusively relied on SSR markers for connecting the individual maps of 'Picual' and 'Arbequina'. The 3:1 segregating markers were excluded at this stage of mapping due to their low informativeness which would affect the determination of the correct order among loci. This conservative approach is appropriate in the absence of sufficient number of co-dominant markers and relatively small mapping population size. Nine linkage groups shared at least one SSR marker between 'Picual' and 'Arbequina' maps. These linkage groups were numbered from 1 to 9 and placed adjacent in the Fig. IV.2, showing connections between homologs. When only one marker was shared the orientation of the linkage groups was established arbitrarily. The rests of the linkage, groups, were numbered arbitrarily from 10 to 23 in each parental map, i.e. there is no correspondence between 'Picual' and 'Arbequina' linkage groups coded with the same number. In the 'Arbequina' map, a total of 392 markers (23 SSR and 369 DArT) were mapped to 204 bins in the 23 linkage groups. In the 'Picual' map consisting of 23 linkage groups, two hundred and fifty seven markers (24 SSR and 233 DArT) were mapped to 125 bins. Around 50% of markers were mapped into the same positions as a result of the small size of the mapping population and some degree of marker redundancy. The total distance covered was 1205.1 cM and 1639.3 cM in 'Picual' and 'Arbequina' maps, respectively. Mean map distance between adjacent bins was 9.64 cM in 'Picual' and 8.04 cM in 'Arbequina', and the average size of the linkage group was 52.40 cM and 71.28 cM, respectively.

Fig IV.2. Genetic linkage map of 'Arbequina' (Arb) and 'Picual' (Pic). To the right of 'Arbequina' linkage group and to the left of 'Picual' groups are the marker name, and to the left the distance (centiMorgans) between markers. The DArT markers are indicated by the first four letters (olPt).

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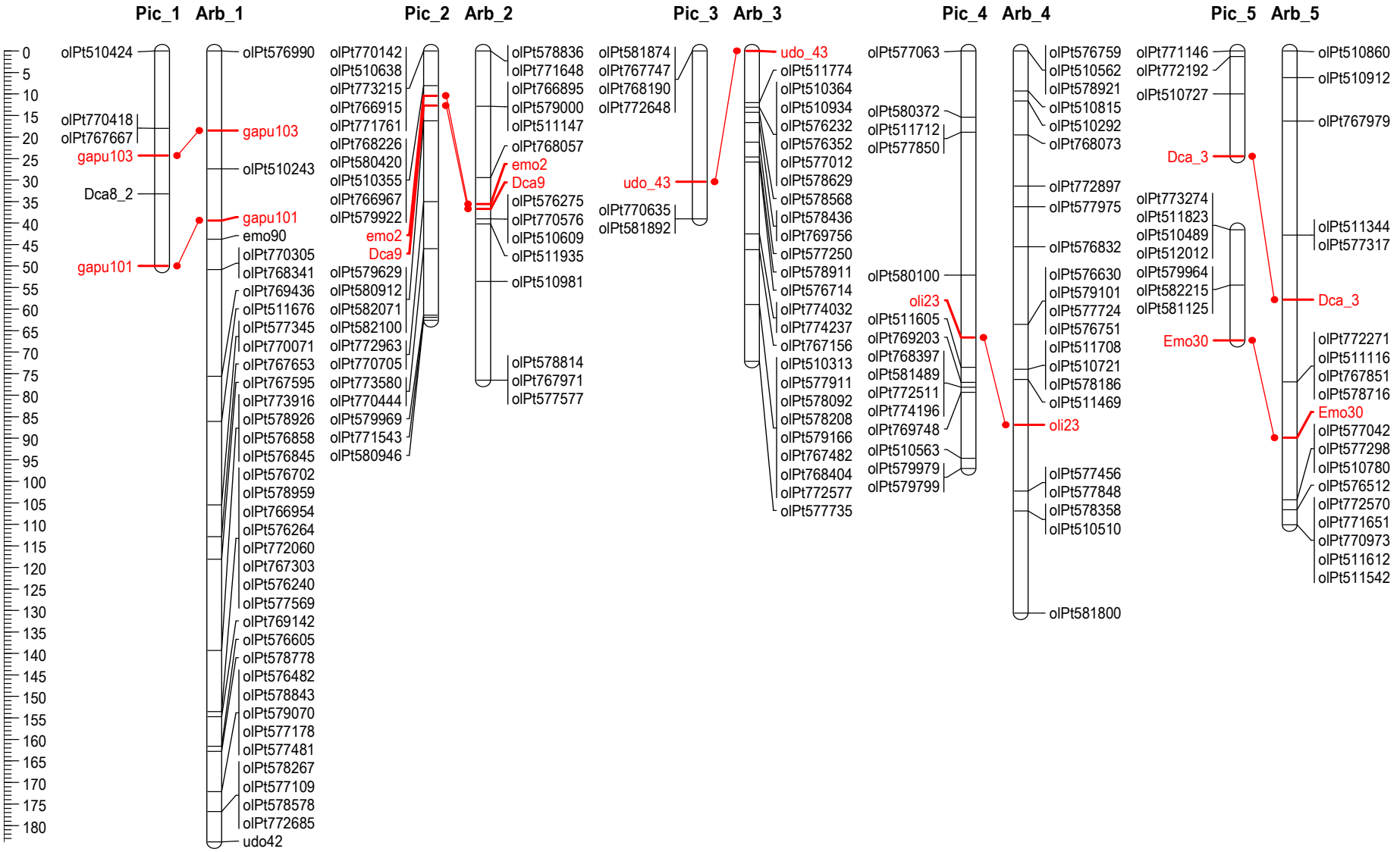
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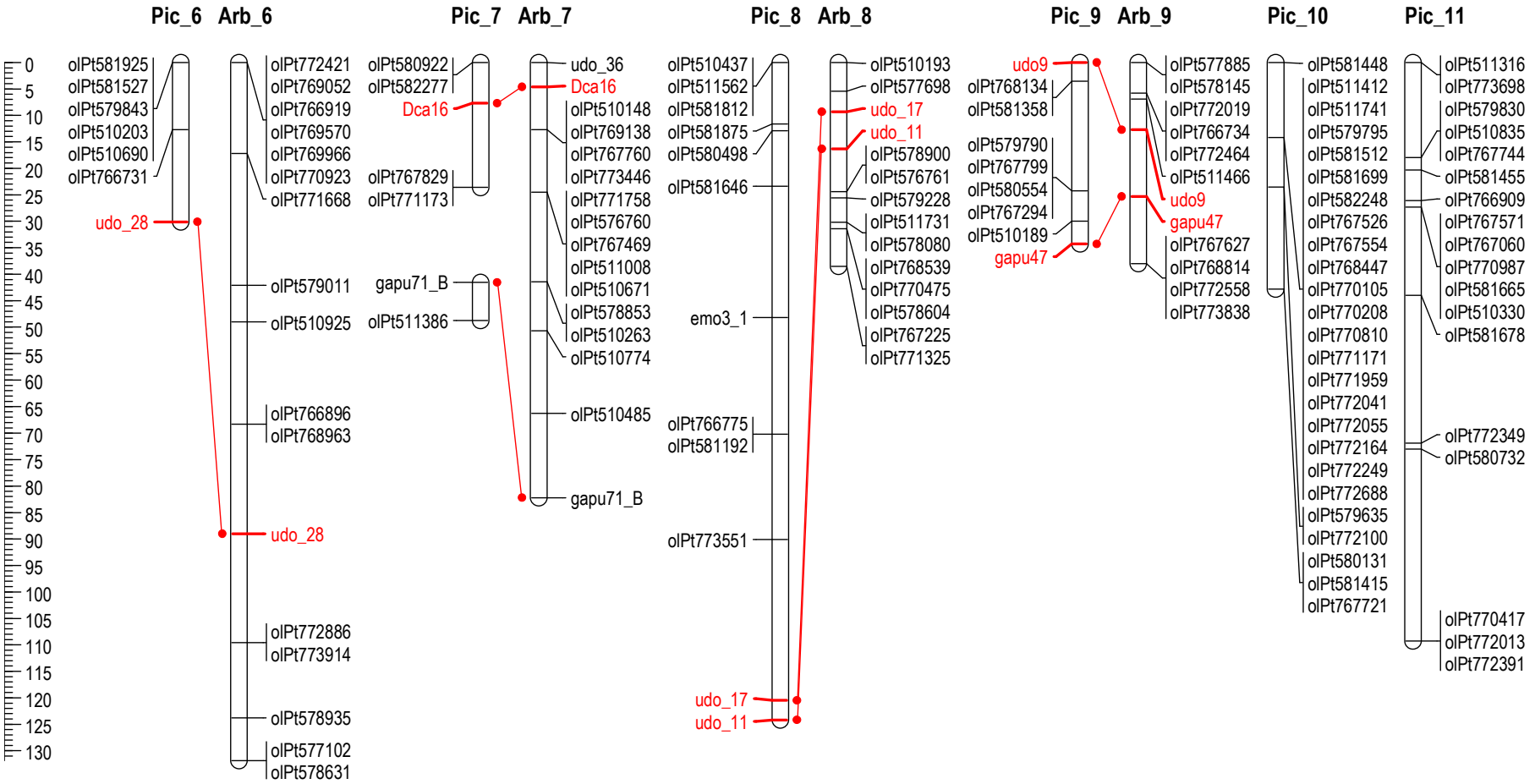
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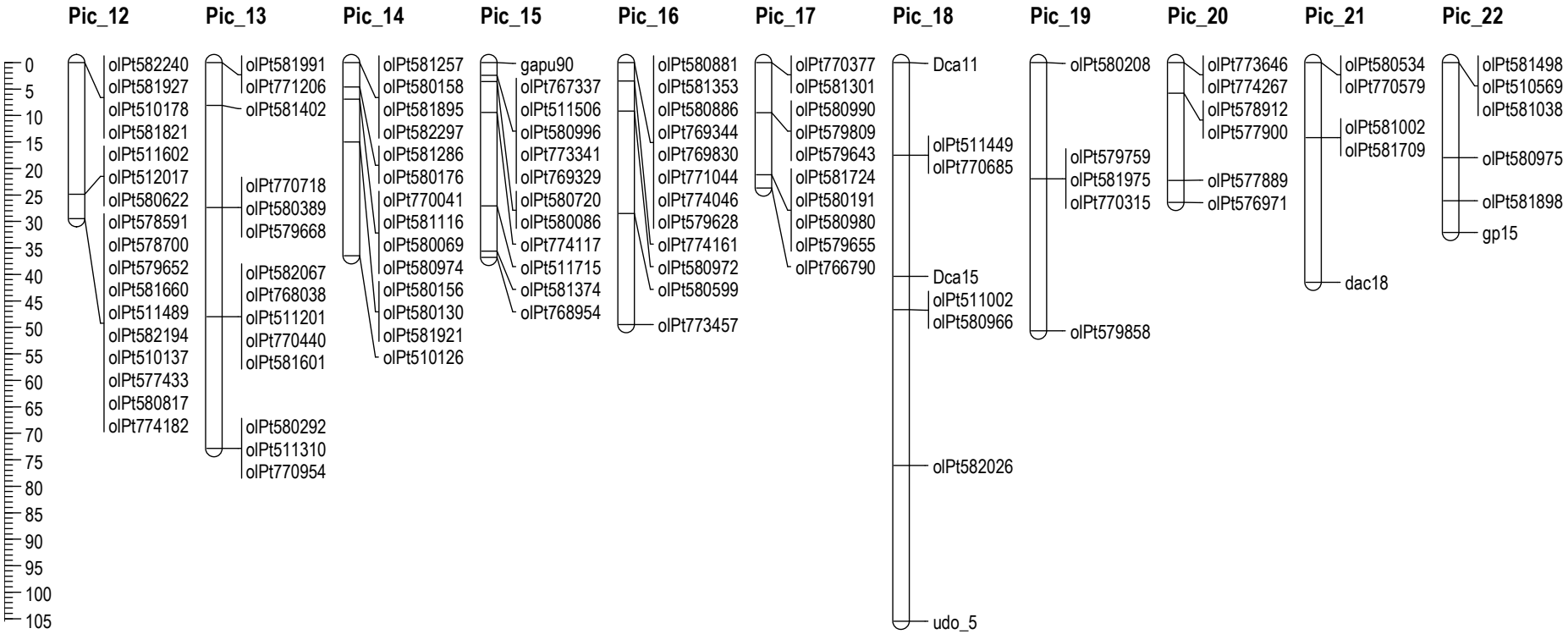
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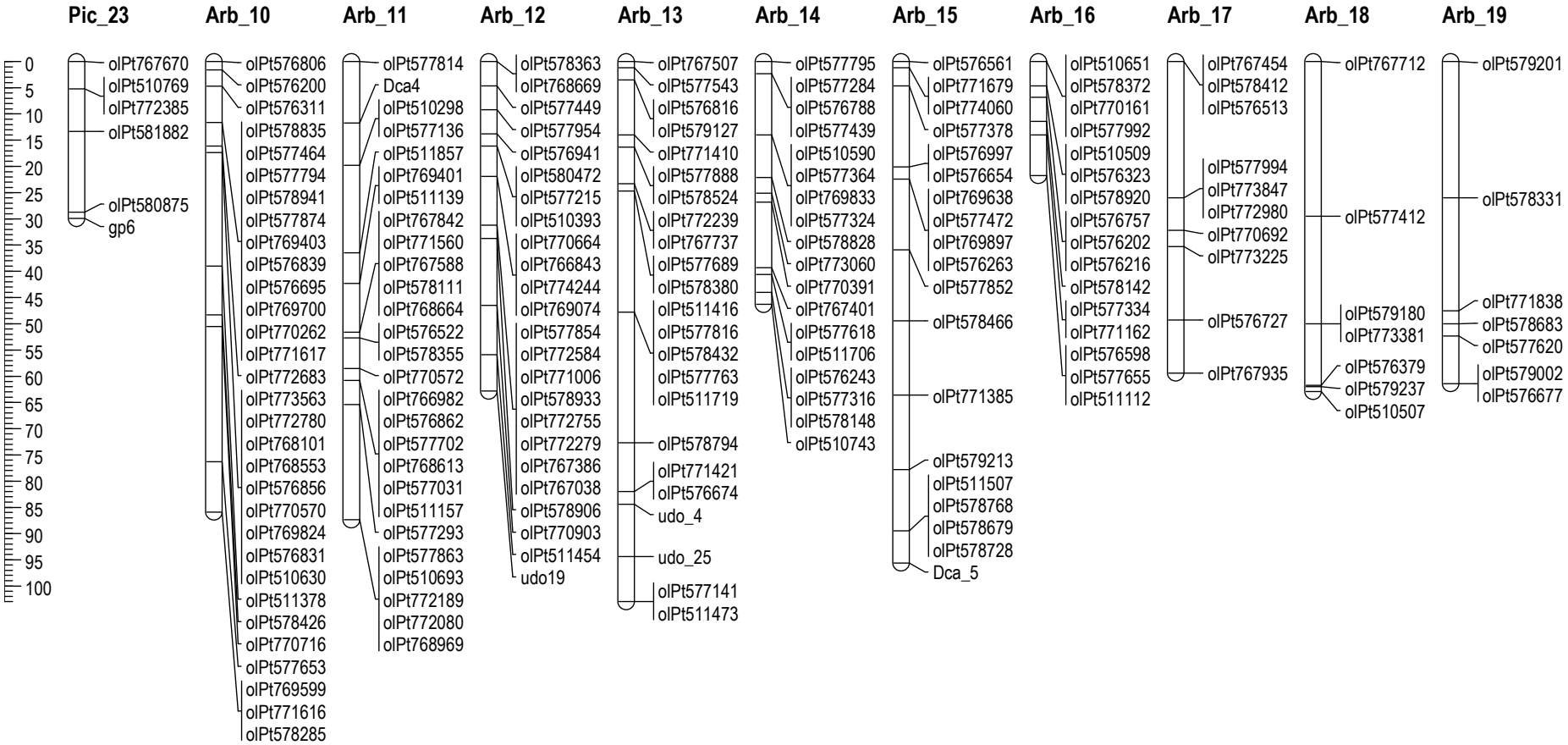
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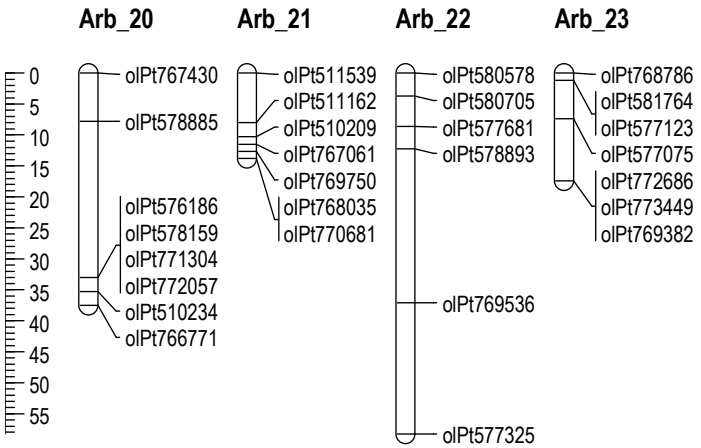
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IV.5. Discussion

IV.5.1. Reproducibility

A significant first step in the development of DArT in a new species is the choice of the complexity reduction method to generate a genomic representation covering the available variability in the species. As expected, the combination *Pst*I/*Taq*I performed well in olive as happens in other species including barley (Wenzl et al., 2004), cassava (Xia et al., 2005), *Arabidopsis* (Wittenberg et al., 2005), wheat (Akbari et al., 2006), *Musa* (Risterucci et al., 2009) and even one of the most challenging crops (due to the highly polyploid genome)-sugarcane (Heller-Uszynska et al, 2010). The complexity reduction method used in this study gave a high polymorphism rate with a good marker quality as revealed by their high PIC values, call rates and P values. PIC values were similar to those obtained in cassava (Hurtado et al., 2008; Xia et al, 2005) a bit lower than in *Musa* (Risterucci et al., 2009), maybe because in this latter case, different species from the genus *Musa* were genotyped. The reproducibility of DArTs was 99.8% which is similar to previous reports in barley (Wenzl. et al., 2004), wheat (Akbari et al., 2006), *Arabidopsis* (Wittenberg et al., 2005), cassava (Xia et al., 2005) and pigeonpea (Yang et al., 2006). Reproducibility is an important issue in olive since even SSRs have to be selected with care to avoid lack of reproducibility between different laboratories (Baldoni et al., 2009).

IV.5.2. Diversity analysis

One of the criteria for selecting genetic markers to be used for fingerprinting and marker-assisted selection is a high level of polymorphism (Jaccoud et al., 2001). The genotyping of DArT markers in a set of cultivars has shown a high range of variability as expected in an outcross species and thus confirming the great diversity previously found within the cultivated olive germplasm (Bartolini et al., 2005). Previous studies in olive with AFLPs (Bandelj et al., 2004) has shown similar PIC values, while SSR markers have shown higher PIC values

(Belaj et al., 2004; Diaz et al., 2007; Khadari et al., 2004; Poljuha et al., 2008) as expected given the codominant nature of the latter.

Due to the nature of olive as an allogamous tree species, Shannon's diversity index values were also high. The values found here were comparable to the ones found in cultivated and wild olives (Belaj et al., 2011a, b), *Malus siversii* population (Zhang et al., 2007) and wheat (Peng et al., 2009).

The mean of effective number of alleles per assay was similar to those found by AFLPs in olive but lower than the ones found in SSRs, probably due to their dominant nature and it may suggest the presence of many unique or less frequent alleles (Belaj et al., 2003). In comparison to the two above mentioned DArT is a high-throughput, quick, cheaper and high reproducible technology. However these markers do share some features with AFLPs, such as their origin, dominant nature and/or the large numbers of markers (Agrawal et al., 2008).

DArT markers permitted the unambiguous identification of all 62 genotypes confirming thus their utility for identification studies, an important issue for the management of germplasm collections (Belaj et al., 2001, 2003) and olive breeding programs (Díaz et al., 2006). As dominant, bi-allelic markers, DArTs provide less information per locus than SSRs. However, this high-throughput technology compensates this fact with a much higher number of loci available which allows better genome coverage and a much lower cost per datapoint.

IV.5.3. Genetic relationships among olive cultivars

Our study shows that DArT markers provide important information regarding the genetic relationships and their resolving power is very good as described in other species (Bolibok-Bragoszewska et al., 2009; Heller-Uszynska et al., 2010; Jing et al., 2009; Mace et al., 2008; Risterucci et al.,. 2009; Tinker et al., 2009; Zhang et al., 2009).

Consistent with previous studies using AFLPs (Rao et al., 2009), SSRs (Cipriani et al., 2002; Diaz et al., 2006), RADPs (Durgac et al., 2010; Hagidimitriou et al., 2005), SCARs (Hernandez et al*.,* 2001a, 2001b; Shu-Biao et al., 2004), ISSRs (Hess et al., 2000; Vargas and Kadereit, 2001) and SNPs (Reale et al., 2006; Rekik et al., 2010), the cluster analysis revealed that the 62 genotypes examined showed a clear demarcation of the germplasm.

The DArT profiles revealed a clustering of cultivars originating from the same or nearby geographic areas as well as a relatively clearly distinction of cultivars from Eastern and Western Mediterranean areas. Our results agree with previous studies which suggest that domestication process might have happened simultaneously at both ends of the Mediterranean (Breton et al., 2006; Lumaret et al., 2004; Terral et al., 2004). Besnard et al. (2000) and Besnard and Bervillé (2000) obtained the same conclusion with chloroplastic and mitochondrial DNA since different mitotypes were associated with Eastern and Western Mediterranean Basin. These results reinforce the hypotheses of multilocal selection and breeding of olive cultivars into their respective areas of origin (Belaj et al., 2002; Besnard et al., 2001; Koehmstedt et al., in press; Owen et al., 2005). In addition, the non-random association of DArTs is most likely the reflection of linkage disequilibrium which could be expected after the many “founder” effects which occurred during the various steps of olive tree domestication in diverse countries as shown previously (Besnard et al., 2001).

The clear clustering of the cultivars originating from the same country such as Turkey, Syria, Albania and Croatia suggests a common genetic base for these cultivars as already proposed (Belaj et al., 2002). Some of the associations between the cultivars were also found in previous studies. For instance, similarly to our study close relationships between the cultivars ‘Picudo’ and ‘Manzanilla Cacereña’ were detected by means of AFLP markers (Belaj et al., 2003) while ‘Cipressino’ and ‘Istarska bjelica’ cultivars clustered together when the same type of markers was used (Bandelj et al., 2004). Previous studies (Belaj et al., 2003) have shown the utility of dominant markers for revealing genetic relationships among cultivars. Interestingly, Koehmstedt et al. (in press)

obtained the same clustering of ‘Arbequina’ and ‘Arbosana’ with SSRs; being the last one considered a descendent of ‘Arbequina’ (J. Tous, personal communication). The clustering of the two Albanian cultivars (‘Klon 14’ and ‘Ulliri i Kuq’) may indicate an autochthonous origin of cultivars from this country (Belaj et al., 2003).

IV.5.4 Utility of DArT in olive genetic mapping

In this study, a preliminary linkage map was developed in a ‘Picual’ × ‘Arbequina’ progeny based on DArTs and a limited set of SSRs. The heterozygous nature of olives species constitutes a serious limitation for genetic mapping when only dominant markers are available. Accordingly, two separate maps have been developed for ‘Picual’ and ‘Arbequina’ as in other linkage maps developed in olive (Baldoni et al., 1999; De la Rosa et al., 2003). To developed the map, we used the pseudo-test cross strategy (Grattapablia and Sederoff, 1994) and we relied in the new maximum likelihood mapping algorithm implemented in JoinMap 4.0 since multipoint analysis is more powerful with markers that are not fully informative and in the presence of missing data (Cartwright et al., 2007). Although dominant markers segregating 3:1 have been used for the development of an integrated map instead of two separated maps in different species including olive (Atienza et al., 2002; Chafari et al., 2009; De Keyser et al., 2010; Wu et al., 2004), we were unable to obtain an integrated map of sufficient quality and thus these markers were not considered at this stage. Nevertheless, they may prove useful when a larger set of co-dominant markers is genotyped in our mapping population. As far as the positioning of SSR markers on the two genetic maps, similarly to previous studies in fruit tree species (Dirlewanger et al., 1998) and olive (Khadari et al., 2010), some of them (36.9%) were genetically linked. While, Khadari et al.(2010) found that 50% of the SSRs used were genetically linked. It is also interesting to mention that in the latter study two markers (EMO2 and Dca9) linked together in the same linkage group as in our case.

Segregation distortion is defined as a deviation of the observed genotypic frequencies from their expected values. It was found in both maternal and

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parental data sets although it was much higher in 'Arbequina', the pollen donor of our mapping population. Similar levels of segregation distortion have been reported in olive (Wu et al., 2004) although it is cross dependant since other authors have reported a level of segregation distortion around 7% at $P < 0.05$ which is near the expected values to be reported due to chance alone (De la Rosa et al., 2003). At the same time the distortions found here were comparable with those reported in other crops, e.g., 17% and 24% for apple (Conner et al., 1997), 10% for chestnut (Cassasoli et al., 2001), 13% for apricot (*Prunus armeniaca* L.) (Hurtado et al., 2002).

The low size of the mapping population might affect the test for distortion. However, a lower population size was used for mapping in an inter-specific cross in *Eucalyptus* and it did not result in significant segregation distortion (Grattapablia and Sederoff, 1994). Thus, the population size effect on segregation distortion can be obviated in our case. In 'Arbequina', the number of markers excluded due to segregation distortion was nine fold compared to 'Picual'. Furthermore, considering a total of 613 markers, around 30 of these are expected to be reported as distorted due to chance alone at $P<0.05$, and thus it may be concluded that the distortion observed in 'Arbequina' has a biological basis. It can be caused by a variety of mechanisms including competitive fertilization; pollen tube competition; pollen abortion or zygotic selection (reviewed by Xian-Liang et al. 2006). Our data suggest that there is a distortion factor(s) in the pollen donor which should be studied more carefully in the future.

The parental maps obtained have a map length higher than the one reported by Wu et al. (2004), similar to El Aabidine et al. (2010) and smaller than in the other maps (De la Rosa et al., 2003; Khadari et al., 2010). As in previous linkage maps, average distance between markers is higher in the female than in the male linkage map. This could be caused by differences in the recombination frequencies between the two parents (Khadari et al., 2010) but it could also reflect a lower level of polymorphism in 'Picual'. Indeed, the number of polymorphic markers useful for 'Arbequina' mapping was near 50% higher than

for 'Picual' mapping. In any case, forthcoming genotyping of this mapping population with SNPs markers that are currently being identified by sequencing in the OLEAGEN project (Valpuesta, personnal communication) and in other sequencing projects (Galla et al., 2009) would help to reduce the percentage of markers unlinked or associated in small groups. Besides, the increase in co-dominant markers may allow using markers segregating in 3:1 proportion which would result in better genome coverage.

In conclusion, we report the first high throughput marker set developed in olive. DArT markers obtained in olive demonstrated a good quality, as assessed by their call rate and scoring reproducibility, comparable to those obtained in other species. Given their relative low cost per data they constitute a powerful tool for olive studies at present and should be useful in diversity studies, QTL discovery and LD mapping in olive.

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CAPÍTULO V

Developing a core collection of olive (*Olea europaea* L.) based on molecular markers (DArTs, SSRs, SNPs) and agronomic traits

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Developing a core collection of olive (*Olea europaea* L.) based on molecular markers (DArTs, SSRs, SNPs) and agronomic traits

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V.1. Abstract

Molecular markers (SSR, SNP and DArT) and agronomical traits have been used in the world's largest olive (Olea europaea L.) germplasm collection (IFAPA, Centre Alameda del Obispo, Cordoba, Spain) to study the patterns of genetic diversity and underlying genetic structure among 361 olive accessions. In addition the marker data were used to construct a set of core collections by means of two different algorithms (MSTRAT and Power-Core) based on M (maximization) strategy. Our results confirm that the germplasm collection is a useful source of genetically diverse material. We also found that geographical origin is an important factor structuring genetic diversity in olive. Subsets of 18, 27, 36, 45 and 68 olive accessions, representing respectively 5%, 7.5%, 10%, 12.5% and 19% of the whole germplasm collection, were selected based on the information obtained by all the data set as well as each marker type considered individually. According to our results, the core collections that represent between 19% and 10% of the total collection size could be considered as optimal to retain the bulk of the genetic diversity found in this collection. Due to its high efficiency at capturing all the alleles/traits states found in the whole collection, the core size of 68 accessions could be of special interest for genetic conservation applications in olive. The high average genetic distance and diversity and the almost equal representation of accessions from different geographical regions indicate that the core size of 36 accessions, could be the working collection for olive breeders.

Keywords: Olive germplasm . Genetic diveristy . Core sets, Molecular markers . Olive breeding

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V.2. Introduction

In most crops, much emphasis has been laid on the collection and conservation of genetic resources leading to the establishment of ex situ germplasm banks around the world. Fundamental goals of a germplasm bank are to acquire, maintain, document, assess and make accessible representative plant genetic diversity of the crop of concern. However, in spite of the remarkable progresses achieved in the last decades to avoid genetic erosion of many crop species, there is still a large gap between the available germplasm diversity collected and its effective use (Van Hintum et al. 2000). The large size, heterogeneous structure and unavailability of information on trait diversity hinder the successful use of the genetic potential of most of these germplasm collections (Brown 1989a, b; Van Hintum et al. 2000). Effective management, research and utilization of the existing variation in these large collections could be greatly enhanced by constituting subsamples also known as core collections or core subsets. In fact, a core collection is a subset of accessions from the entire collection that capture, with minimum redundancy, most of the available genetic diversity of a crop, a wild species or a group of species (Brown 1989a, b; Van Hintum et al. 2000). Although the size of the core subset depends mainly on the global amount of the accessions available, it should always be substantially smaller than the collection from which it was formed. To date, most core collections developed contain 5–20% of the accessions conserved in the whole germplasm collection with no redundant entries (Van Hintum et al. 2000). A core subset should provide conclusions transposable to the entire collection as well as make it easier to look for allelic variation in genes of interest and more efficient to asses genotype–phenotype associations (Balfourier et al. 2007; Richards et al. 2009).

Development of core collections has traditionally been based on passport and phenotypic data (Upadhyaya et al. 2001; Amalraj et al. 2006). However, biochemical (Chandra et al. 2002) and DNA markers, such as RAPDs (Marita et al. 2000), AFLPs (Van Treuren et al. 2006), SSRs (Balfourier et al. 2007; Escribano et al. 2008; Richards et al. 2009; Ronfort et al. 2006; Volk et al. 2005)

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and SNPs (Le Cunff et al. 2008; McKhann et al. 2004), are increasingly being used for this purpose either alone and/ or in combination with phenotypic traits (Richards et al. 2009; Volk et al. 2005). The significant advances achieved in the assessment of genetic diversity of germplasm collections have also been accompanied by the development of different approaches and bioinformatic tools used to guide the construction of core collections (Franco et al. 2005, 2006; Marita et al. 2000; Schoen and Brown 1993). These different approaches have been compared in annual (Franco et al. 2006) and perennial species (Escribano et al. 2008). Both studies concluded that the maximization (M) strategy, i.e., the one that maximises the number of alleles at each locus (Schoen and Brown 1993), is the most powerful approach that maintains the most diverse alleles, eliminates redundancy and captures most of the genetic diversity of the whole collection. In contrast to other approaches previously used, the M strategy does not rely on a priori stratified sampling of the larger collections (Schoen and Brown 1993, 1995). Although it was initially based on marker variation, the M strategy can also be applied to qualitative and quantitative variables (Gouesnard et al. 2001).

The majority of studies that deal with the development of core sets have been performed in annual species (Balfourier et al. 2007; McKhann et al. 2004; Ronfort et al. 2006). However, the advantages of building core collections are particularly desirable for woody perennial species that are usually maintained as clones in field genebanks (Escribano et al. 2008; Richards et al. 2009). Higher management costs and vulnerability to environmental conditions are the most distinctive features of field germplasm collections in comparison to seed germplasm banks. Besides, the domestication process of woody perennial species has usually involved few recombination cycles and consequently only a small number of generations separates the domesticated genotypes from their wild ancestors, being so the population structure of perennial species significantly different from that of annual ones (Escribano et al. 2008). Development of core collections has been performed in a number of perennial and fruit tree species, such as cashew (Dhanaraj et al. 2002), sandalwood (Shashidhara et al. 2003), wild apple (Richards et al. 2009; Volk et al. 2005),

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grape (Barnaud et al. 2006; Cipriani et al. 2010; Le Cunff et al. 2008), pear (Miranda et al. 2010) and cherimoya (Escribano et al. 2008).

However, up to date, no core collection has been developed in any olive (Olea europaea L.) germplasm collection. This evergreen fruit tree species, autochthonous of the Mediterranean basin, plays a peculiar role in the landscape characterization and represents the sixth most important edible oil crop worldwide. Key attributes of olive include its relatively small (2,200 Mb) diploid (2n=46) genome, its predominantly allogamous nature and its wide genetic patrimony, represented of thousands of local and old cultivars, restricted to specific areas where they originally grew (Barranco and Rallo 2000; Bartolini et al. 2005; De la Rosa et al. 2003; Loureiro et al. 2007). The richness of the cultivated olive germplasm represents an unusual case among horticultural crops, as a consequence of tree longevity and lack of turnover with new breeding genotypes. More than 1,200 varieties are still under cultivation, 79 international and national collections located in 24 countries hold about 4,200 genotypes and more than 5,300 cultivar names are recognised (Bartolini et al. 2005). This great genetic patrimony has been evaluated by means of traditional methods (Barranco et al. 2005), isozymes (Trujillo et al. 1995) and DNA molecular markers (Belaj et al. 2001, 2003; Besnard et al. 2001a; Cipriani et al. 2002; Muzzalupo et al. 2009; Sarri et al. 2006). However, there is still a limited knowledge on the real variability present in olive germplasm collections and only a restricted number of well-known cultivars have been employed for olive breeding purposes (Rallo et al. 2008). Establishing a core subset of an olive collection could facilitate the evaluation and utilization of its genetic diversity for crop improvement.

In this sense, this work attempts to develop a core collection representative of the diversity conserved in the World Olive Germplasm Bank (WOGB) located in Córdoba, Spain. Started in 1970, this collection is an international reference on olive germplasm due to the high number of accessions included and their high degree of identification and evaluation (Barranco and Rallo 2000; Belaj et al. 2001; Rallo et al. 2005). For that purpose, we first aimed at quantifying the

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genetic diversity and underlying genetic structure of accessions from the WOGB by means of agronomical traits, simple sequence repeats (SSR), single-nucleotide polymorphism (SNP) and Diversity Array Technology (DArT) molecular markers. In a second step, the results of these analyses were used to generate a group of core subsets based on the M strategy and, in the final step, the diversity parameters of the core subsets were compared to the entire collection in order to select the best ones. Possible applications of the selected core sets in breeding, genomic and variability studies are discussed.

V.3. Material and methods

V.3.1. Plant material

The WOGB maintained at IFAPA Alameda del Obispo in Cordoba (Andalusia, Southern Spain) was used as plant material supplier for this study. The WOGB was initiated in 1970 by a FAO project by the auspices of the International Olive Oil Council (IOOC). The collection includes cultivars collected from Spanish and international prospecting surveys and/or provided by different scientific institutions, some of them participating in EUIOOC Resgen projects. Nowadays the WOGB represents the largest repository of cultivated material in the world (Barranco et al. 2005; Belaj et al. 2001; Caballero et al. 2005). From this collection, 361 cultivars (see online resource 1) were chosen based on their identification level and availability of both agronomic and genotyping data. This plant material includes cultivars from 19 different countries, including Albania (8), Algeria (2), Chile (1), Croatia (6), Cyprus (4), Egypt (5), France (8), Greece (12), Iran (5), Israel (3), Italy (22), Lebanon (1), Morocco (3), Portugal (8), Spain (205), Syria (39), Tunisia (7), Turkey (19) and the United States of America (3).

V.3.2. Agronomic characterization of the WOGB

The agronomic characterization of the WOGB cultivars was performed during two consecutive harvesting seasons, 2008/2009 and 2009/2010. This included four characters related to vigour (tree height, trunk cross section, canopy surface and volume) and three related to fruit characteristics (fruit weight, flesh

stone ratio and fruit oil content). Fruit characteristics were measured in homogenous samples of 1 kg as described by Del Río et al. (2005a). The agronomic data from the seven traits were converted to categorical variables based on the classification described previously in the WOGB (Del Río et al. 2005a, b).

V.3.3. Molecular characterization of the WOGB

V.3.3.1. DNA extraction and microsatellite genotyping

Total DNA was extracted from fresh leaves following the protocol described by De la Rosa et al. (2002). A set of 23 microsatellites loci: DCA1, DCA4, DCA5, DCA8, and DCA10 (Sefc et al. 2000), EMO-9 (De la Rosa et al. 2002), GAPU-11e17, GAPU-45, GAPU-71A, GAPU-82, GAPU- 89, and GAPU-108 (Carriero et al. 2002) GP7, GP11 and GP15 (Sabino-Gil et al. 2006) UDO4, UDO5, UDO12, UDO15, UDO17, UDO27, UDO31 and UDO42 (Cipriani et al. 2002) were used for genotyping accessions from the whole collection. The selection and use of this set of SSR markers was prior to the work published by Baldoni et al. (2009). Although less polymorphic than the loci selected in that study (Baldoni et al. 2009), these primers, previously tested in a representative sample of 48 cultivars, did not show any problems of reliability and reproducibility within the laboratory (Trujillo et al., unpublished data).

The PCRs were carried out in a thermal cycler GeneAmp PCR system 9600 (Applied Biosystems) with an initial denaturation at 94℃ for 5 min, followed by 35 cycles of 94℃ for 20 s, the annealing temperatu re according to the author instructions, for 30 s and 72℃ for 30 s, and final extension at 72℃ for 7 min. PCR products were separated using an automatic capillary sequencer (ABI 3130 Genetic Analyzer Applied Biosystems/HITACHI) at the Unit of Genomics of the Central Service for Research Support of the University of Córdoba, Spain. The softwares Genescan v. 3.7 and Genotyper v. 3.7 from Applied Biosystems were used for sample analysis. Three reference samples were used in all runs.

V. 3.3.2. SNP genotyping

The seven SNPs used for genotyping (Cycl-1, Cycl-2, Lup, Ant-1, LS5, Cbp and LS2) were described by Reale et al. (2006). These SNPs were the only available at the beginning of this study and had also successfully been used in identification studies (Dominguez-García et al., submitted). DNA extraction for SNP analysis was also based on the method described by De la Rosa et al. (2002). Polymerase chain reactions were performed following the conditions and compositions described by Reale et al. (2006). SNPs were verified by using the SNaP-shotTM multiplex system. PCR products were loaded on an ABI Prism 3100 (Applied Biosystems) using POP-4 separation polymer at standard running conditions at the Unit of Genomics of the Central Service for Research Support of the University of Cordoba. The softwares Genescan v. 3.7 and Genotyper v. 3.7 (Applied Biosystems) were used for sample analysis and peak sizes determination.

V.3.3.3. DArT markers

Four subgenomic libraries were constructed using PstI/TaqI DArT genomic complexity reduction methods as described by Akbari et al. (2006). DNA from 93 olive cultivars, likely covering a great part of the variability available at the WOGB, were used to generate DArT libraries substantially as described by Jaccoud et al. (2001). Genomic DNA was extracted from young leaves according to the method described by De la Rosa et al. (2002) with minor modifications. The libraries generated from the genomic representations contained in a total of 15,360 clones. Inserts from all these clones were amplified, purified and arrayed on microarray slides as described by Wenzl et al. (2004). Slides were scanned using a Tecan LS300 (Grödig, Slazburg, Austria). and array images were analysed with DArTsoft v. 7.4 (Diversity Arrays Technology P/L, Canberra, Australia). Markers were scored “1” for presence or “0” for absence in the genomic representation, and “X” when the DArTsoft was unable to score the sample with sufficient confidence. DArTsoft was used to automatically analyse batches of up to 96 slides to identify and score

polymorphic markers. The threshold criteria of reproducibility and call rate were set to be higher than 97% and 80%, respectively. Redundancy of the polymorphic DArT clones was evaluated with the software package DArT ToolBox (http://www.diversityarrays.com/), and approximately 30% of the potentially redundant markers were maintained, similarly to what has been done in Eucalyptus (Sansaloni et al. 2010). A more detailed description on the development of DArT genotying arrays and selection of markers for diversity analysis is being given elsewhere (Dominguez-García et al., submitted). Both array development and DArT genotyping were performed using service from Diversity Arrays Technology Pty Ltd (Canberra, Australia). A total of 1,255 markers were used in the present study.

V.3.4.Data analysis

V.3.4.1. Genetic diversity of the whole collection

Genetic diversity of the whole collection was analysed by calculating the total (Na) and average number of alleles (Navg), effective number of alleles (NE; Berg and Hamrick 1997) and Shannon's diversity index (Sh; Lewontin 1972) for each type of markers. For agronomic traits (treated as qualitative data), total and average number of states per trait as well as the effective number of states and Shannon's diversity index were estimated by using the same formulae as in case of molecular markers where allele frequencies were replaced by state frequencies.

For codominant markers (i.e., SSRs and SNPs), the observed (HO) and expected heterozygosity or gene diversity (HE) were calculated using PowerMarker v. 3.25 (Liu and Muse 2005). All the diversity parameters used are based either on a simple count (Na, Npr, HO) or depend solely on allelic frequencies (NE, Sh, HE) and can be applied without assuming the Hardy–Weinberg equilibrium. Three Mediterranean regions were established considering the countries of origin of the cultivars and previous studies in olive (Belaj et al. 2002; Sarri et al. 2006): (1) East Mediterranean (Cyprus, Egypt,

Iran, Israel, Lebanon, Syria and Turkey), (2) central Mediterranean (Albania, Algeria, Croatia, Greece, Italy and Tunisia) and (3) west Mediterranean including New World cultivars (Chile, France, Morocco, Spain, Portugal and USA). Genetic diversity estimates (Navg, NE and Sh for all marker types; HO and HE for codominant markers only) based on all data as well as separately for each marker type were compared among regions. Repeated measures analysis of variance was carried out using PROC GLM in SAS (SAS Institute 2004). Post hoc Bonferroni's adjustments were used to compare the means of diversity estimates among regions at significance level $P<0.05$.

V.3.4.2. Analysis of the genetic structure of the WOGB

To assess the structure of the genetic diversity within the entire collection, three different approaches were used: graphic clustering by means of principal coordinate analysis (PCoA), the analysis of molecular variance (AMOVA) and a model-based clustering method using STRUCTURE analysis. PCoA analysis based on Dice's distance matrix calculated from DArT, SSR and SNP markers was performed, using NTSYS-pc v. 2.10 (Rohlf 2005). in order to graphically represent genetic relationships among olive cultivars. Dice's distance coefficient between olive cultivars was obtained as 1-Dice's similarity index (Dice 1945) using NTSYS-pc. Pearson correlation coefficient and Mantel's test (Mantel 1967) were used to compute and test the linear correlation between Dice's distance matrices based on DArTs and SSRs. The significance level was assessed after 10,000 permutations in NTSYS-pc.

The AMOVA (Excoffier et al. 1992) using ARLEQUIN v. 2.0 (Schneider et al. 2000) was based on Dice's distance matrix. AMOVA was used to partition the total molecular diversity (A) among and within regions (Eastern, Central and Western Mediterranean) and (B) among regions, among countries within regions, and within populations. The variance components were tested statistically by nonparametric randomisation tests using 10,000 permutations.

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A model-based clustering method was applied to all molecular data (SSRs, SNPs and DArTs) to infer genetic structure and to define the number of clusters (gene pools) in the dataset using STRUCTURE v, 2.2.3 (Falush et al. 2003; Pritchard et al. 2000). Given a value for the number of clusters, this method assigns individual genotypes from the entire sample to clusters in a way in which linkage disequilibrium is maximally explained. This method has been successfully applied to identify clusters of genetically similar accessions of a range of cultivated plants as maize (Liu et al. 2003), peas (Jing et al. 2010) and olives (Belaj et al. 2010). Ten runs of STRUCTURE were performed by setting the number of clusters (K) from 1 to 11. Each run consisted of a burn-in period of 20,000 steps followed by 100,000 Monte Carlo Markov Chain replicates, assuming an admixture model and correlated allele frequencies. No prior information was used to define the clusters. The choice of the most likely number of clusters (K) was carried out by comparing the average estimates of the likelihood of the data, ln[Pr(X|K)], for each value of K (Pritchard et al. 2000), as well as calculating an ad hoc statistic ΔK based on the rate of change in the log probability of data between successive K values, as described by Evanno et al. (2005). The proportions of membership (Q) of each individual in each gene pool were also calculated.

V.3.4.3. Construction of the core collection

Two different algorithms based on M (maximization) strategy were used to form core collections. The standard M strategy described by Schoen and Brown (1993) was employed as implemented in MSTRAT (Gouesnard et al. 2001). MSTRAT algorithm selects a subset of n accessions from the N accessions of the entire collection by maximizing the number of alleles at each locus (as well as states of each trait). Four core subsets with different sizes were developed including 18 (5% of the entire collection), 27 (7. 5%), 36 (10%) and 45 (12.5%) accessions. For each sampling size, 200 independent replicates and 100 iterations were generated and the core subset having the highest Shannon's diversity index was chosen.

The *advanced* M (maximization) strategy using heuristic search as proposed by Kim et al. (2007) was carried out as implemented in PowerCore v. 1.0. PowerCore selects the accessions with higher diversity representing the total coverage of marker alleles and trait states present in the entire collection. Thus, the size of the final core collection is not known a priori and it depends on the levels of variability and redundancy present in the collection.

In both cases, six reference cultivars (also called kernel cultivars) were included (Gouesnard et al. 2001). The kernel cultivars were chosen based on their productivity importance in olive-growing countries as well as their use into olive breeding programs. They were: 'Koroneiki' (Greece), 'Frantoio' and 'Leccino' (Italy), 'Arbequina', 'Manzanilla de Sevilla' and 'Picual' (Spain).

V.3.4.4. Validation of the representativeness of the core collections

Total number of alleles/states and coverage of alleles (percentage of alleles/states from the entire collection which are also present in the core subset) as well as genetic diversity measures (Navg, NE and Sh for all marker types; HO and HEfor codominant markers only) based on all markers as well as separately for each marker type were estimated for each core subset. Repeated measures analysis of variance was carried out using PROC GLM in SAS (SAS Institute 2004). Post hoc Bonferroni's adjustments were used to compare the means of diversity estimates from different core subsets with the entire collection used as control.

V.4. Results

V.4.1. Genetic diversity and underlying genetic structure in an olive collection

Diversity parameters (Navg, average number of alleles/states; NE, effective number of alleles and Sh, Shannon's diversity index) for the set of marker analysed in the WOGB collection are summarized in Table V.1. The first two axes of PCoA analysis based on Dice's distances calculated from DArTs, SSRs and SNPs (Fig. V.1) accounted for 16.54% and 9.89% of the total variance, respectively. The low percentage of variance explained by these two first

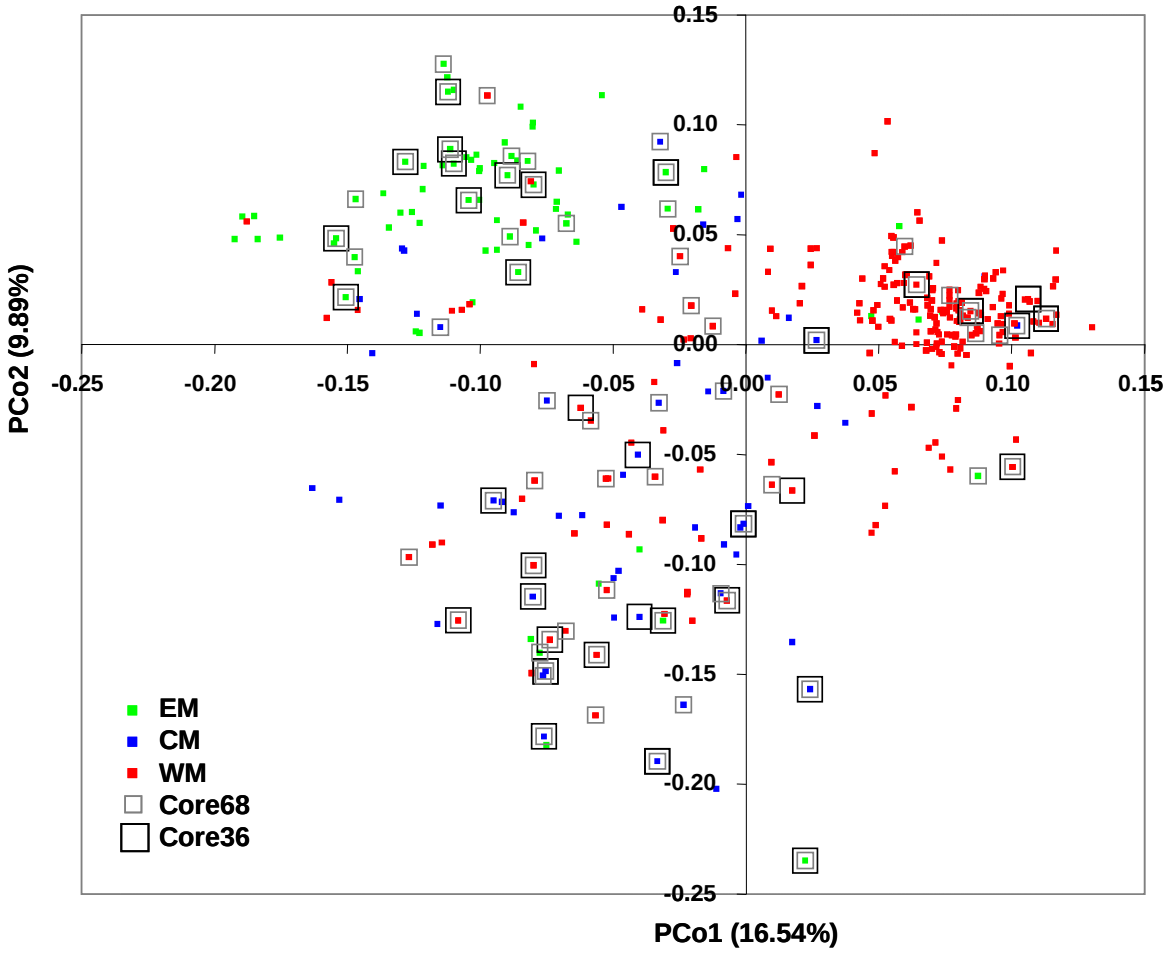
components indicates that the large number of variables used in the present study cannot be efficiently reduced to planar representation.

Tab V.1. Diversity parameters of the entire collection for the markers used

Parameter	All markers	DArTs	SSRs	SNPs	Agronomic traits
N_a	2,816	3	261	14	31
N_{avg}	2.180	2.000	11.348	2.000	4.429
N_E	1.600	1.560	3.066	1.817	3.637
H_o	-	-	0.525	0.463	-
H_E	-	-	0.623	0.442	-
Sh	0.519	0.500	1.318	0.632	1.351

N_a total number of alleles/states, N_{avg} average number of alleles/states, N_e, effective number of alleles/states, H_O observed heterozygosity, H_E expected heterozygosity, Sh Shannon's diversity index

Fig V.1. Principal coordinate analysis of 361 olive accessions based on Dice's distance calculated from DArT, SSR and SNP markers. Each accession is coloured depending on their regions of origin eastern Mediterranean (EM), centra Mediterranean (CM) and western Mediterranean (WM). Accession included in cores 68 and 36 are indicated by *small and big squares*, respectively.



However, even with such significant loss of information, a clustering by geographical origin is evident in the PCoA graph. The first axis separated the majority of olive accessions from the west Mediterranean region from those belonging to the central and east Mediterranean regions. Along the second PCo axis, the majority of accessions from the central Mediterranean region clustered separately from those belonging to the east Mediterranean one. Average pairwise Dice's distances obtained by DArTs (0.28) were lower than those based on SSR data (0.48), and the correlation between the distance matrices based on these two markers was highly significant (r=0.69, P<0.0001).

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One-way AMOVA (Table V.2) showed that most of the genetic diversity was attributable to differences among accessions within regions (90.36%). In fact, ϕst value among regions was significant (P<0.001) although very weak considering the low percentage of variance. Similarly, in the two-way AMOVA, most of the diversity was found within countries (86.78%) while the remaining variability was almost equally distributed among regions (5.66%) and among countries/within regions (7.56%).

Tab V. 2. AMOVA analysis for the partitioning of the total molecular diversity

Analysis	Source of variation	df	Variance components	Percentage of variation	ϕ-statistics	P(ϕ)
(A)	Among regions	2	0.014	9.64	ϕ_{ST} = 0.096	< 0.0001
	Within regions	358	0.133	90.36		
(B)	Among regions	2	0.008	5.66	ϕ_{CT} = 0.057	< 0.0001
	Among countries within regions	16	0.011	7.56	ϕ_{SC} = 0.080	< 0.0001
	Within countries	342	0.129	86.78	ϕ_{ST} = 0.132	< 0.0001

(A) among and within regions (Eastern, Central and Western Mediterranean) and (B) between regions, among countries within regions, and within populations.
P(ϕ) - ϕ-statistics probability level after 10,000 permutations

The STRUCTURE analysis indicated K=2 (ΔK=461.93) as the most likely number of clusters (hereafter called gene pools) while the second-best solution was K=3 (ΔK= 173.70). The proportions of membership (Q) of each individual in each gene pool were calculated (Fig. V. 2; online resource 1).

At K=2 the gene pool A is predominant across western Mediterranean accessions with the average proportion of genome assigned to this gene pool at Q=76% (Fig V. 2). The accessions from Spain, Morocco and Portugal were mainly assigned to this gene pool at Q=77%, 99% and 91%, respectively. The gene pool B occurred in accessions from eastern Mediterranean (Q=81%) as well as from central Mediterranean regions (Q=71%). Syrian (Q=86%), Iranian (Q=99%), Albanian (Q=85%) and Italian accessions (Q= 73%) were among the ones with the highest proportion assigned to this gene pool.

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For both K=2 and 3, some accessions were assigned to a gene pool different from their region of sampling (Fig. V.2; online resource 1). For instance, olive accessions from France and Israel were almost equally assigned to the eastern and western Mediterranean regions. On the contrary, it is interesting to notice that most Iranian and Syrian accessions along with some Turkish ones were always assigned to their region of origin (eastern Mediterranean) with a proportion of membership higher than 99%. Finally, the central Mediterranean region was the most admixed one with accessions assigned to each of the three gene pools having proportions of membership (Q) higher than 75%.

Fig V.2. Structure of olive accessions from WOGB obtained with DArT, SSR and SNP markers. Each accession is represented by a single vertical line divided into colours. Each colour represents one gene pool, and the length of the coloured segment shows the individual's estimated proportion of membership in that gene pool. Gene pool A mainly associated with western Mediterranean region is indicated in *red.* While *green* indicates gene pool B associated with eastern and central regions.

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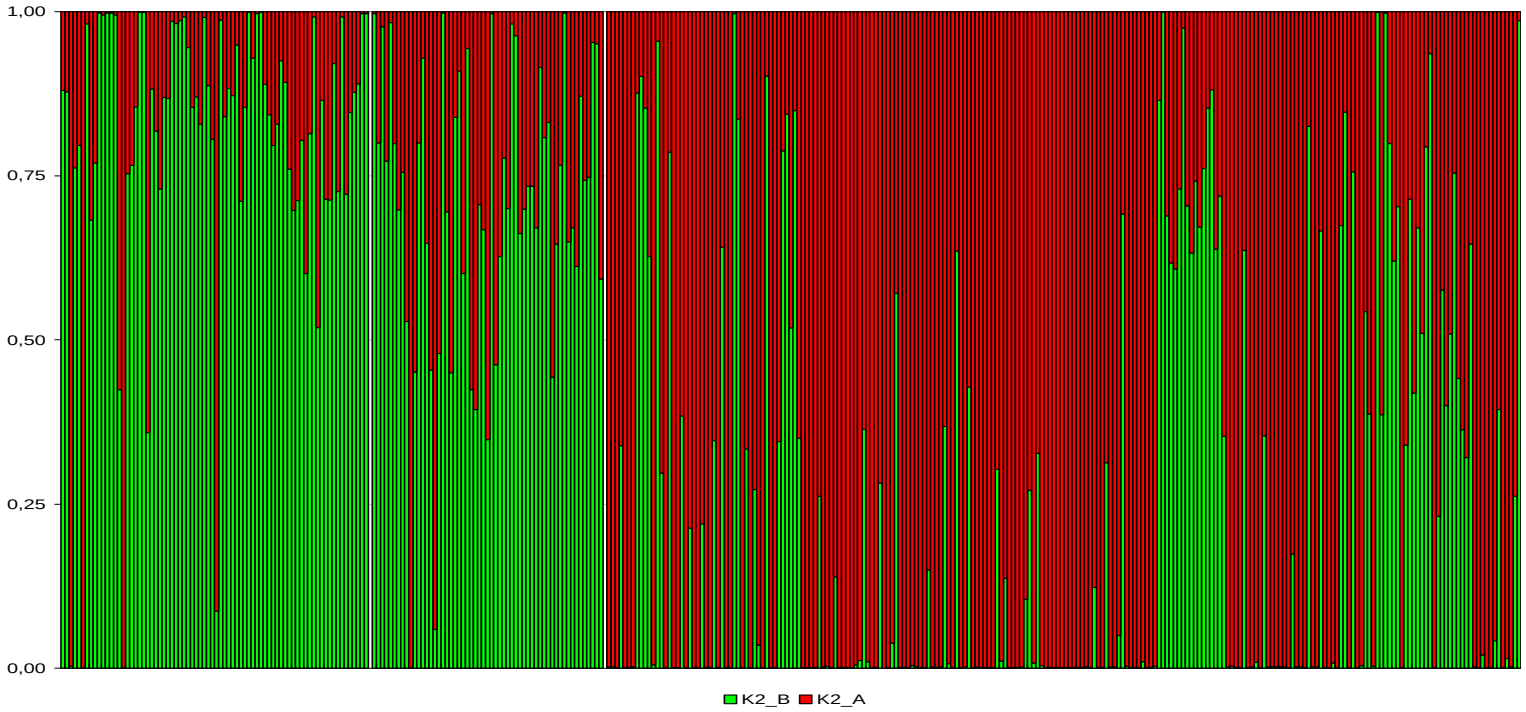
K=2



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V.4.2. Developing of core subsets and comparison to the entire collection

Four different core collections were obtained by means of the MSTRAT program. These core collections were composed of 5% (18 accessions), 7.5% (27 accessions), 10% (36 accessions) and 12.5% (45 accessions) of the entire collection (Table V.3; online resource 2). The core collection obtained by the PowerCore, representing a full coverage of all the alleles (and trait states) existing in the entire collection, was composed of 68 accessions (19%). In addition to the kernel cultivars, only eight accessions were shared by all the core subsets while 21 accessions were shared by core collections 68, 45 and 36 (online resource 2). The different core collections will hereafter be named after the number of accessions selected in each case, i.e., core 18, core 27, core 36, core 45 and core 68.

When all types of markers were jointly used, a high percentage of the total number of alleles (Na) detected in the entire collection was maintained by the core collections (Table V.3). As expected, 100% of Na detected in the entire collection was maintained by the core 68 while it ranged from 96% (core 18) to 99% (cores 45 and 36) for the MSTRAT core collections. Cores 27 and 18 showed significantly lower values ($P<0.001$) of the average number of alleles (Navg) than the entire collection. In all core subsets, significantly higher values ($P<0.001$) for the effective number of alleles (NE) and Shannon's diversity index (Sh) were obtained in comparison with the whole collection. The highest values of these parameters were obtained by core 36 (NE=1.69; Sh=0.564). Finally, higher average pairwise Dice's genetic distances were obtained by all core subsets in comparison to the entire collection (data not shown).

Tab V.3. Diversity parameters for the different core subsets compared to the entire collection.

Marker type	Parameter	Entire collection	Core subsets									
			Core68		Core45		Core36		Core27		Core18	
All markers	N_a (T %)	2816	2816	(100)	2792	(99)	2778	(99)	2748	(98)	2709	(96)
	N_{avg}	2.180	2.180		2.161		2.150		2.127	***	2.097	***
	N_e	1.600	1.668	***	1.681	***	1.686	***	1.677	***	1.672	***
	Sh	0.519	0.557	***	0.564	***	0.564	***	0.559	***	0.556	***
DArTs	N_a (T%)	2510	2510	(100)	2510	(100)	2510	(100)	2507	(100)	2494	(99)
	N_{avg}	2.000	2.000		2.000		2.000		1.998		1.989	***
	N_e	1.560	1.611	***	1.624	***	1.626	***	1.623	***	1.620	***
	Sh	0.500	0.534	***	0.541	***	0.541	***	0.536	***	0.534	***
SSRs	N_a (T%)	261	261	(100)	237	(91)	223	(85)	196	(75)	171	(66)
	N_{avg}	11.348	11.348		10.304		9.696	*	8.522	***	7.435	***
	N_e	3.066	4.127	***	4.188	***	4.367	***	4.078	***	3.967	**
	H_O	0.525	0.531		0.527		0.529		0.540		0.528	
	H_E	0.623	0.686	***	0.692	***	0.696	***	0.698	***	0.693	***
	Sh	1.318	1.559	***	1.576	***	1.580	***	1.548	***	1.505	***
SNPs	N_a (T%)	14	14	(100)	14	(100)	14	(100)	14	(100)	14	(100)
	N_{avg}	2.000	2.000		2.000		2.000		2.000		2.000	
	N_e	1.817	1.813		1.792		1.751		1.777		1.757	
	H_O	0.463	0.417		0.391	***	0.359	***	0.379	***	0.357	***
	H_E	0.442	0.445		0.437		0.422		0.433		0.424	
	Sh	0.632	0.636		0.628		0.611		0.623		0.613	
Agronomic traits	N_a (T%)	31	31	(100)	31	(100)	31	(100)	31	(100)	28	(90)
	N_{avg}	4.429	4.429		4.429		4.429		4.429		4.000	***
	N_e	3.637	3.781		3.652		3.510		3.386		3.378	
	Sh	1.351	1.379		1.358		1.323		1.305		1.267	

N_a (T%), total number of alleles/states and coverage of alleles (% of alleles/states from the entire collection which are also present in the core subset); N_{avg}, average number of alleles/states; N_e, effective number of alleles/states; H_O, observed heterozygosity; H_E, expected heterozygosity; Sh, Shannon's diversity index. [#]Significant differences as compared to the entire collection: "***" $P < 0.001$, "**" $0.001 < P < 0.01$, "*" $0.01 < P < 0.05$, non-significant values not indicated

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When each marker type was taken individually, all core collections still maintained most of the alleles found in the whole collection (ranging from 66% to 100%). As expected, core 68 maintained 100% of the alleles for each of the four different marker types. Core 18 showed significantly lower values of Navg than the whole collection for all markers but SNPs. Significantly higher values of NE and Sh were retained in all the core subsets, by means of DArTs and SSRs, in comparison with the whole collection. Core 36 showed the highest values for NE and Sh indexes for those two marker types. When using SNPs and agronomic traits, no significant differences were found between the core subsets and the entire collection for NE and Sh. Core 68 showed the highest values for those parameters.

Observed heterozygosity (HO) values retained by means of SSRs in the core subsets were similar to the whole collection, cores 27 and 68 being the ones which showed the highest values. In the case of expected heterozygosity (HE), significantly higher values were recorded for all the core subsets, core subsets 27 and 36 being the ones that showed the highest values. A significant drop of HO values was observed for all core collections, except core 68, when SNPs were used. In contrast, for the same marker, no significant differences were found between the core subsets and the whole collection for HE values. Cores 68 and 36 showed the highest values of this parameter.

All core subsets displayed a relatively good representation of accessions from each Mediterranean region (Fig. V.1; Table V.4; online resource 2). The eastern Mediterranean region was the best represented (33.8–44.4%) in spite of the fact that 63.2% of the accessions of the whole collection correspond to the western area. It should be noted that cores 36 and 27 retained a balanced representation (more than 30%) of accessions from each Mediterranean region.

Tab V.4. Number of countries and accessions per region represented in the entire collection and the core collections

Set	Region									Total	
	Eastern			Central			Western				
	No. Countries	No. Acc.	%T	No. Countries	No. Acc.	%T	No. Countries	No. Acc.	%T	No. Countries	No. Acc.
Entire	7	76	21.05	6	57	15.79	6	228	63.16	19	361
Core68	6	23	33.82	6	17	25.00	2	28	41.18	14	68
Core45	5	17	37.78	5	11	24.44	2	17	37.78	12	45
Core36	4	13	36.11	4	11	30.56	1	12	33.33	9	36
Core27	4	10	37.04	4	9	33.33	1	8	29.63	9	27
Core18	4	8	44.44	3	5	27.78	1	5	27.78	8	18

%T, % of total number of accessions

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Regarding the representation of countries from Mediterranean regions, core 68 retained accessions from most of the countries found in the WOGB (14 out of 19). Surprisingly, a very low proportion of western Mediterranean countries (one or two out of the six found in WOGB) were retained by all the subsets. In this sense both eastern (four to six out of seven countries) and central (three to six out of six countries) Mediterranean countries were well represented into the core subsets in comparison to the WOGB collection, but Spain and Syria were the countries with most representation into the different core subsets. This was expected since these two countries are over-represented in the WOGB. Overall, cores 68 and 36 (Table V.5) showed best adjustment with the validation parameters of the representativeness of the whole collections.

Tab V.5. Olive cultivars included in the core 36 and core 68

Cultivar	R.N.	Country	Area of difusion
Abbadi Abou Gabra-842[a]	842	Syria	East
Abou Satl Mohazam[a]	1,04	Syria	East
Abou Kanani[a]	840	Syria	East
Aggzi Shami-1	723	Egypt	East
Arbequina[a]	231	Spain	West
Arbosana	666	Spain	West
Barnea[a]	711	Israel	East
Barri[a]	1,03	Syria	East
Belluti	690	Turkey	East
Blanqueta-48	48	Spain	West
Canetera	653	Spain	West
Caninese	77	Italy	Centre
Carrasqueño de Porcuna	795	Spain	West
Klon-14-1812[b]	1,81	Albania	Centre
Shami-1041	1,04	Syria	East
Chemlal de Kabylie[a]	118	Algeria	Centre
Chemlali	744	Tunisia	Centre
Shengeh[a]	1,14	Iran	East
Dokkar[a]	539	Turkey	East
Empeltre	13	Spain	West
Figueretes	822	Spain	West
Fishomi	1,14	Iran	East
Forastera de Tortosa[a]	652	Spain	West
Frantoio A. Corsini	81	Italy	Centre
Frantoio[a]	80	Italy	Centre
Gaydoyrelia	695	Grecce	Centre

Table V.5. (Continued) Olive cultivars included in the core 36 and core 68

Cultivar	R.N.	Country	Area of difusion
Grappolo	181	Italy	Centre
Grosal de Cieza	802	Spain	West
Istarska Bjelica	735	HRV	Centre
Jabali[a]	1,12	Syria	East
Joanenca	667	Spain	West
Kalamon[a]	105	Grecce	Centre
Kato Drys	848	Cyprus	East
Kelb et Ter-144	144	Syria	East
Khashabi	1,12	Syria	East
KIranz	679	Turkey	East
Koroneiki[a]	218	Grecce	Centre
Ulliri i Kug	1,078	Albania	Centre
Leccino[a]	82	Italy	Centre
Lucques	322	France	West
Llumeta[a]	226	Spain	West
Maarri[a]	1,125	Syria	East
Manzanilla de Almeria	784	Spain	West
Manzanilla de Sevilla[a]	21	Spain	West
Manzanilla de Tortosa	22	Spain	West
Manzanillera de Huecal Overa[a]	757	Spain	West
Mari[a]	1,143	Iran	East
Masabi	1,121	Syria	East
Mastoidis[a]	345	Grecce	Centre
Mavreya[a]	699	Grecce	Centre
Majhol-1013[a]	1,013	Syria	East
Majhol-152[a]	152	Syria	East
Megaritiki[a]	108	Grecce	Centre
Menya[a]	669	Spain	West
Mixani	1,079	Albania	Centre
Morejona	349	Spain	West
Morrut[b]	224	Spain	West
Myrtolia[a]	700	Grecce	Centre
Palomar	661	Spain	West
Pendolino	87	Italy	Centre
Picual[a]	9	Spain	West
Picudo[b]	3	Spain	West
Picholine-70	70	France	West
Piñonera[a]	969	Spain	West
Redondilla de Logroño	16	Spain	West
Tanche	74	France	West
Tempranoa	358	Spain	West
Toffahi	721	Egypt	East

Table V.5. (Continued) Olive cultivars included in the core 36 and core 68

Cultivar	R.N.	Country	Area of difusion
Uslu[a]	95	Turkey	East
Verdial de Badajoz-877	877	Spain	West
Verdial de Velez-Malaga	883	Spain	West
Verdial de Velez-Malaga-1[a]	51	Spain	West
Zarzariega de Orcera	356	Spain	West

Counties and Mediterranean areas where those cultivars are widely grown as well as their R.N. in the World Olive Germplasm Bank of Cordoba, Spain are so indicated
R.N. register number
[b] Cultivars only included in core 36
[a] Cultivars included in both core sets and those only included in core 68 have no specific mark.

V.5. Discussion

V.5.1. Genetic diversity and underlying genetic structure in an olive collection

Here we describe a survey of a worldwide olive collection (361 accessions) for a set of agronomic and molecular (DArTs, SSRs, SNPs) markers, the largest and most extensive study of this kind to date. The amount of genetic variability revealed in the collection was expectable in an outcrossing, perennial and clonally propagated species such as olive tree. High levels of diversity has also been found in olive, either among cultivars of a single country (Muzzalupo et al. 2009) as well as in representative sample sets deriving from different countries (Belaj et al. 2002; Besnard et al. 2001a; Breton et al. 2006; Koehmstedt et al. 2010; Sarri et al. 2006). However, a certain risk of reduction of local genetic resources and the need of its conservation have also been evidenced (Baldoni et al. 2006; Belaj et al. 2010).

The low percentages of variance explained by the two first components of PCoA analysis are consistent with similar studies and reflect the high dimensionality of the data, as a consequence of a high number of unlinked markers (Leigh et al. 2005). The STRUCTURE and PCoA analyses revealed a certain clustering of the majority of olive accessions according to their regional origin, being the accessions from eastern and western Mediterranean the best differentiated ones. Our findings reinforce the hypotheses of a simultaneously

domestication process at both ends of the Mediterranean (Breton et al. 2006; Lumaret et al. 2004; Terral et al. 2004) where multilocal selection and breeding of olive cultivars might have occurred (Belaj et al. 2002; Besnard et al. 2001a; Koehmstedt et al. 2010; Owen et al. 2005). The preferential clustering of most of accessions from the central Mediterranean region with the eastern Mediterranean cultivars (PCoA; K=2), the high levels of genetic diversity found in this region (in spite of the relatively low number of accessions in the collection), and the assignment of some of its accessions into an independent gene pool (PCoA; K=3) may suggest various scenarios for the olive origin in this region: (1) an east to west dispersal pattern of olive cultivars with human migration (Baldoni et al. 2006; Besnard et al. 2001a, b; Koehmstedt et al. 2010; Terral et al. 2004); (2) a possible local selection of wild genotypes best adapted to environmental conditions and to agronomic expectations (Belaj et al. 2010; Breton et al. 2006; Hannachi et al. 2008) and (3) a further breeding of cultivars introduced from abroad with local material either wild and/or cultivated (Belaj et al. 2002; Besnard et al. 2001a; Sarri et al. 2006). The highest number of private alleles and the highest percentage of correctly assigned accessions found in the eastern Mediterranean region indicates that a number of alleles distinctive for this gene pool have been maintained. Finally, our results are in total agreement with previous studies (Belaj et al. 2002; Besnard et al. 2001a; Sarri et al. 2006), which have found that geographical origin may be an important factor structuring genetic diversity in olive. Local adaptation can explain some differences between accessions and could be of great interest for olive breeding.

V.5.2. Selection of core subsets and comparison to the entire collection

Despite the high variability found in the WOGB, the development of a core collection capable to represent its variability in a smaller number of accessions could be desirable for olive breeding and scientific research. Similarly to other fruit tree species (Cipriani et al. 2010; Escribano et al. 2008; Le Cunff et al. 2008; Miranda et al. 2010), a low sampling intensity (ranging from 5% to 19% of the whole collection) was sufficient to efficiently represent the genetic diversity found in the whole collection. In this sense, when all types of markers were

used, the five olive core collections obtained in this study retained a good allelic coverage (ranging from 96% to 100%), showed significantly higher effective number of alleles and genetic diversity and had higher average genetic distance compared with the whole collection. And only in the case of SSR markers, the decreasing number of genotypes included in each core set was accompanied by a significant loss of alleles. The good representation of the three Mediterranean olive-growing regions as well as the inclusion of kernel cultivars of great agronomical relevance could give an added value to the core subsets from the breeders' point of view. However, the reduced size of the core subsets indicates a certain redundancy and/or high genetic similarity of olive accessions maintained in the WOGB collection. In fact, although in the whole collection, western accessions from Spain and eastern ones from Syria are much more represented than the rest, the three Mediterranean areas are evenly represented in all the core subsets obtained.

The core collections that showed the best adjustment with the validation parameters of the representativeness of the whole collections were core 68 (developed by PowerCore) and the MSTRAT core 36. The similarities found between the PowerCore and MSTRAT methods indicate that the algorithms used by both these methods are very similar.

As expected, core 68 successfully captured all of the alleles and agronomic traits existing in the whole collection. Being selected with a relatively high sampling intensity (19% of the whole collection) and due to its high efficiency in capturing all the alleles/traits states and preserving the private alleles, this core set could be an appropriate choice for applications involving genetic conservation in olive. Another characteristic of PowerCore is that the same list of selected olive accessions should be generated if the selection based on the same data set would be repeated (Kim et al. 2007).

Although obtained with a smaller sampling intensity (10% of the whole collection), core 36 also showed a 99% coverage rate of the alleles/traits states from the entire collection (i.e., 85% for SSRs, and 100% for DArTs, SNPs and

agronomic traits). Both cores 36 and 68 effectively retained observed and expected heterozygosity values obtained by codominant markers in comparison with the whole collection. Besides, 75% of accessions from core 36, including the kernel cultivars, were shared by both core subsets. The high average genetic distance and diversity represented in a smaller number of accessions than core 68 and the almost equal representation of accessions from the three olive-growing Mediterranean regions indicate that core 36 would be the working collection for olive breeders and olive breeding programs. At the same time, this core collection provides useful information for a better management of the WOGB collection. In fact, high level of redundancy and an over-representation of similar and/or redundant accessions from western Mediterranean region have been observed. The good representation of olive accessions from the eastern and central Mediterranean region in core 36, in spite of their lower number in the germplasm bank, suggests that ongoing research and management projects should be aimed at enriching the WOGB collections with olive cultivars from these two regions. In addition to the above-mentioned characteristics of the selected core 36, we also found that its allelic/state frequencies were significantly correlated at high values with the whole collection. This indicates that not only were the same alleles represented in this core subset but they did it at similar frequencies.

By maximising the genetic diversity in a reduced number of genotypes, core 36 could facilitate the study of the variability and correlation of morphologic and agronomic traits. Additionally, core 36 represents an ideal set of genotypes for supporting ongoing efforts of olive genomics and sequencing, validation of new molecular markers as SNPs and exploration of their associations with agronomic traits of interest for breeders. The detailed evaluations of this core collection may also be useful for the choice of optimal parents for olive breeding programs and for generating QTL mapping populations. Due to the perennial and vegetative propagated nature of olive, core 36, developed in this study, could easily be disseminated for comparative trials and other research activities.

In conclusion, we believe that the approach followed in this work could be efficiently applied to develop core subsets in olive and other fruit tree species collections. However, a good core set should be dynamic and revised periodically as additional accessions are incorporated into the whole collection. Finally, the development of core collections in other species (Balfourier et al. 2007; McKhann et al. 2004; Richards et al. 2009) has evidenced that core sets maximised for diversity using a set of specific attributes (molecular or phenotypic), at the same time, maximises unknown diversity. In this sense, future studies may also give interesting insights about the representation of unknown olive diversity (morphological traits, resistance to diseases and pests, olive oil quality parameters, etc.) into the selected core subsets in comparison to the whole collection.

V.6. Acknowledgements

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CAPÍTULO VI

Conclusiones

1. Los marcadores microsatélites y SNP fueron adecuados para explorar la variabilidad genética encontrada en las variedades de olivo argelinas. Sin embargo, los marcadores SNP mostraron una capacidad discriminante menor que los microsatélites (Characterization and identification of the main algerian olive cultivars by molecular markers (2012), Journal of Horticultural Science and Biotechnology, 87 (2); 95-100).

2. A pesar del bajo número de muestras estudiadas, los resultados sugieren una alta variabilidad genética en el germoplasma de olivo de Argelia, que anteriormente había sido poco explorado (Characterization and identification of the main algerian olive cultivars by molecular markers (2012), Journal of Horticultural Science and Biotechnology, 87 (2); 95-100).

3. Los marcadores DArT desarrollados se han revelado útiles para la identificación y estudios de relaciones genéticas en olivo (Development of DArT markers in olive (*Olea europaea* L.) and usefulness in variability studies and genome mapping (2012), 136, 50-60).

4. Se ha comprobado la utilidad de los DArTs para la construcción de mapas de ligamiento, a pesar de su naturaleza dominante y bialélica (Development of DArT markers in olive (*Olea europaea* L.) and usefulness in variability studies and genome mapping (2012), 136, 50-60).

5. El uso de marcadores moleculares (DArT, SSR y SNP) y datos agronómicos ha permitido el desarrollo y la selección de dos colecciones nucleares a partir del Banco de Germoplasma Mundial de Olivo. La colección 68, interesante para estudios de conservación genética; y la colección 36, adecuada para programas de mejora. En este sentido, la estrategia de maximización se ha revelado muy eficiente para la

elaboración de colecciones nucleares en olivo (Developing a core collection of olive (*Olea europaea* L.) based on molecular markers (DarTs, SSRs, SNPs) and agronomic traits (2012), Tree Genetic and Genomes 8, 365-378).

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6. Los casos de homonimias hallados tanto en el nuevo germoplasma introducido de Argelia como en el material existente en la colección, indican la necesidad de realizar un estudio en profundidad de la variabilidad del germoplasma encontrado en el BGMO (Characterization and identification of the main algerian olive cultivars by molecular markers (2012), Journal of Horticultural Science and Biotechnology, 87 (2); 95-100; Development of DArT markers in olive (*Olea europaea* L.) and usefulness in variability studies and genome mapping (2012), 136, 50-60).

7. Todos los marcadores utilizados han demostrado su utilidad en la identificación, estudios de diversidad, manejo de bancos de germoplasma y mejora de olivo (Characterization and identification of the main algerian olive cultivars by molecular markers (2012), Journal of Horticultural Science and Biotechnology, 87 (2); 95-100; Development of DArT markers in olive (*Olea europaea* L.) and usefulness in variability studies and genome mapping (2012), 136, 50-60; Developing a core collection of olive (*Olea europaea* L.) based on molecular markers (DArTs, SSRs, SNPs) and agronomic traits (2012), Tree Genetic and Genomes 8, 365-378).

CAPÍTULO VII

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Anexos

OTRAS COMUNICACIONES A CONGRESOS

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